(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 June 2002 (20.06.2002)

PCT

(10) International Publication Number
WO 02/47681 A1

(51) International Patent Classification[7]: A61K 31/365, 31/366, 31/121, 31/19, A61P 31/04

(21) International Application Number: PCT/AU01/01621

(22) International Filing Date:
14 December 2001 (14.12.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PR 2090 14 December 2000 (14.12.2000) AU

(71) Applicant *(for all designated States except US)*: **UNISEARCH LIMITED** [AU/AU]; Rupert Myers Building, Level 2, Gate 14, Barker Street, UNSW, Sydney, New South Wales 2052 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KJELLBERG, Staffan** [SE/AU]; 22 Goorawahl Avenue, La Perouse, New South Wales 2036 (AU). **RICE, Scott** [AU/AU]; 27 George Street, Paddington, New South Wales 2021 (AU). **MCDOUGALD, Diane** [AU/AU]; 27 George Street, Paddington, New South Wales 2021 (AU).

(74) Agent: **FB RICE & CO.**; 139-141 Rathdowne Street, Carlton, Victoria 3053 (AU).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*
— *entirely in electronic form (except for this front page) and available upon request from the International Bureau*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 02/47681 A1

(54) Title: REGULATION OF BACTERIAL VIRULENCE

(57) Abstract: The present invention relates to methods of inhibiting virulence in organisms with an AI-2 system using furanones and related compounds. These methods represent a novel mechanism for controlling disease causing organisms.

# REGULATION OF BACTERIAL VIRULENCE

## FIELD OF THE INVENTION:

The present invention relates to methods of inhibiting bacterial virulence. In particular the present invention relates to methods of inhibiting virulence in organisms with an AI-2 system using a substance that inhibits this system. The substance may be a furanone or related compound. These methods represent a novel mechanism for controlling disease causing organisms. For example, current antibiotics rely on global effects on protein production or on general growth inhibition rather than to prevent the expression of a limited set of genes that are required to establish a productive infection.

## BACKGROUND OF THE INVENTION

*Vibrio* species are widely distributed in aquatic environments, especially in coastal, tropical or temperate waters. *Vibrio vulnificus* is ubiquitous in tropical and temperate oceans and has been isolated from seawater, sediment, plankton, animals, intestines of fish and from bivalves in the U. S. (46) and from areas such as Brazil (54), the Netherlands (68), Denmark (20), India (65) and Korea (56). *V. vulnificus* occurrence is correlated with salinity and temperature (23) with numbers being greater when temperatures are between 13 and 22°C and salinity is between 5 and 25 ppt, although culturable cells have been isolated from mollusks at 7°C (20). At water temperatures below 10°C, the numbers of culturable cells of *V. vulnificus* drops below detection. The loss of culturable cells below 10°C has been demonstrated to be due to the cells entering a viable but nonculturable (VBNC) state (33, 45).

The VBNC state has been suggested to represent a survival strategy, analogous to spore formation, which allows the cells to persist for extended periods under adverse conditions. For example, VBNC cells of *V. vulnificus* have been demonstrated to have increased mechanical stability (48). It has been demonstrated that starvation of *V. vulnificus* prior to shifting cells to low temperatures induces the starvation-induced maintenance of culturability (SIMC) response which delays the induction of the VBNC response (43, 48). This would suggest that starvation-induced or stationary phase genes are

important for the adaptation of this organism to a variety of stress conditions including, but not limited to low temperature survival.

In many bacteria, the regulation of phenotypes is controlled via signalling pathways where the secretion and recognition of small extracellular factors is used to coordinate the expression of phenotypes at the population level. Many of these signal-regulated phenotypes are induced during stationary phase. For example, *Rhizobium leguminosarum* uses diffusible signal molecules to induce stationary phase (13) and conditioned supernatants have been shown to induce carbon-starvation proteins in *Vibrio angustum* (60). Many virulence factors are also induced during the stationary phase of growth, for example, the expression of the metalloprotease of *V. vulnificus* has been demonstrated to be maximal during late exponential phase and was stable during stationary phase (24). Interestingly, in a range of other organisms, signalling molecules have been demonstrated or suggested to regulate the expression of virulence factors (41, 50, 59, 69).

Numerous factors that are important for virulence have been proposed for *V. vulnificus*, such as the expression of an antiphagocytic capsular polysaccharide (CPS) (26, 58), type IV pili (9), and the production of siderophores (28, 57). In addition, this organism produces a number of extracellular proteins including a cytolysin (14, 27), a novel hemolysin (5), a phospholipase and lysophospholipase (64), hyaluronidase, fibrinolysin and chondroitin sulfatase (47). Possibly one of the most important extracellular virulence factors is an elastolytic and collagenolytic metalloprotease (VVP) (25, 40) which has been shown to degrade albumin, immunoglobulin G and complement C3 and C4 (52). In its purified form, this protease has been shown to induce hemmorhagic damage, enhance vascular permeability and edema, and is lethal to mice when administered intraperitoneally or intravascularly (39). In addition, the metalloprotease has been implicated in the release of iron bound to transferrin or lactoferrin (42) which makes the iron available to the siderophore and enhances growth of the bacterium under conditions of low iron. The VVP of *V. vulnificus* has been shown to be homologous to the HA protease (HA/P) of *Vibrio cholerae* (17) which is regulated by HapR (22). This activator protein is homologous to the LuxR protein of *Vibrio harveyi*. In addition, another *luxR* homologue, *opaR*, has been demonstrated to regulate capsule production in *Vibrio parahaemolyticus* (32). These regulatory proteins are members of a signalling system that has

been discovered in an ever increasing number of organisms (1), including both Gram-negative and Gram-positive bacteria. In most cases, the genes regulated by this signalling system are unknown, but regulation of virulence is indicated in *Escherichia coli, Salmonella typhimurium* (59, 62) and *V. cholerae* (22). It has also been demonstrated that the metalloprotease is expressed in stationary phase in *V. cholerae* and *V. vulnificus*, at a time when signalling processes have been shown to play important roles in regulation in other bacteria. Due to the similarities in the metalloproteases of *V. cholerae* and *V. vulnificus* we initiated a search for a *luxR* homologue in *V. vulnificus* and identified the *smcR* gene which is a homologue of the *V. harveyi luxR* gene and *hapR* of *V. cholerae* (34).

SUMMARY OF THE INVENTION

The present inventors have found that the virulence factor of a microorganism can be inhibited by exposing the microorganism to a substance that inhibits a pathway that regulates the expression of the virulence factor. The present inventors have found that furanones, and related compounds are capable of blocking the signalling cascade of the AI-2 signalling pathway, which was originally described for *Vibrio harveyi*. This signalling system has subsequently been described in a range of bacteria, including, but not limited to, *Escherichia coli, Salmonella typhimurium, Vibrio cholerae, Vibrio vulnificus*, and there is evidence that the system is present in a broader range of bacteria, including *Bacillus anthracis, Enterococcus faecalis, Yersinia pestis, Mycobacterium tuberculosis* and *Heamophilus influenza*.

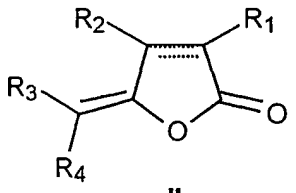
The AI-2 signalling system has been suggested to regulate virulence in a range of pathogens including *V. cholerae*, enteropathogenic (EPEC) and enterohemmorrhagic (EHEC) *E. coli*, *V. vulnificus* and *V. parahaemolyticus*. This would suggest that regulation of virulence phenotypes by AI-2 is common and therefore it seems likely that AI-2 will regulate the expression of virulence factors in organisms with an AI-2 system. The present inventors have directly shown that a range of furanones and related compounds inhibit virulence factor production in *V. vulnificus* and that furanones block AI-2 mediated bioluminescence in *V. harveyi*. Thus, it appears that furanones are capable of disrupting AI-2 mediated gene expression in a range of bacteria and that the effects of the furanones can be logically extended to the range of

bacteria which rely on AI-2 signalling to control the expression of phenotypes that are important for the development of virulence.

In a first aspect, the present invention provides a method of inhibiting a virulence factor in a microorganism comprising exposing the microorganism to a substance that inhibits a pathway that regulates the expression of the virulence factor.

The pathway may be an AI-2 signalling system pathway.

In a second aspect the present invention provides a method of inhibiting a virulence factor in a microorganism, the method comprising exposing the microorganism to a composition comprising an effective concentration of a compound of general formula II:




II

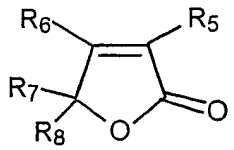
wherein $R_1$ and $R_2$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_3$ and $R_4$ are independently H, halogen, alkyl, aryl or arylalkyl, alkoxy, alkylsilyl; ;

$R_3$ or $R_4$ + $R_2$ can be a saturated or an unsaturated cycloalkane;

and "-------" represents a single bond or a double bond,

or a compound of general formula III:




III

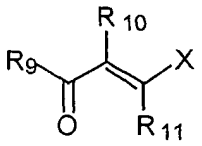
wherein $R_5$ and $R_6$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted,

optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_7$ and $R_8$ are independently H, halogen, hydroxy, alkyl, haloalkyl, dihaloalkyl, trihaloalkyl, aryl, or arylalkyl, a hydroxy compound or a derivative of a hydroxy compound;

$R_7$ or $R_8$ + $R_6$ is saturated or unsaturated cycloalkane,

or a compound of general formula IV




IV

wherein $R_{10}$ and $R_{11}$ are independently H, halogen, carboxyl, ester, formyl, cyano, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;
X is a halogen;

$R_9$ is H, alkyl, alkenyl, alkynyl, alkene, alkyne, aryl, arylalkyl, whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic.

The term "virulence factor" as used herein includes any microbial product that is generally recognised as virulence factors, including those specifically described herein, as well as aspects of microbial physiology that are responsible for causing disease or pathology. For example, the virulence factor may be a specific toxin, secondary metabolite, proteases etc. The physiological factors are those making the microorganism a more effective infectious agent, such as by modifying the host immune response, protecting themselves from the host response or by making the organism more resistant to removal, either by the host response, resistance to therapeutic treatment or cleaning, such as biofilm formation, surface attachment, surface translocation or stress resistance.

In a preferred embodiment of the present invention the virulence factor is an AI-2 mediated phenotype.

In a further preferred embodiment the virulence factor is selected from the group consisting of protease production including exoprotease production, capsule production, biofilm formation, surface colonisation, toxin production and/or secretion, starvation tolerance, VBNC formation, stress survival and combinations thereof.

In one preferred embodiment, the virulence factor is a metalloprotease. It has been demonstrated that the AI-2 system is important for the control of protease production, specifically metalloproteases and we have demonstrated that furanones can inhibit production of these metalloproteases. Non-limiting examples of metalloproteases include VvpR of *V. vulnificus*, HA/P of *V. cholerae*, or EmpA from *V. parahaemolyticus*. Examples of these proteases can be found in a broad range of bacteria including, but not limited to, *Yersinia*, *Mycobacterium*, *Thermoplasma*, *Sulfolobus* and *Bacillus*.

In yet a further preferred embodiment of the present invention, the virulence factor is metalloprotease production and the microorganism is *V. vulnificus*.

The method of the present invention is particularly useful in the treatment of those strains of a particular microorganism that are pathogenic, including those strains that are capable of opportunistic infection, for example, pathogenic strains of *E. coli* such as EHEC or EPEC strains, and O1 and O139 strains of *V. cholerae*.

The microorganism may be one having genes with homology to *luxS*, *luxR* (from V. harveyi), *smcR*, or that are demonstrated to be AI-2 positive in a bioassay, such as the BB170 bioluminescence assay described herein. Examples are: *E. coli*, *Salmonella typhimurium*, *S. typhi*, *S. paratyphi*, *Shigella flexneri*, *Haemophilus influenza*, *Helicobacter pylori*, *Bacillus subtilis*, *Borrelia burgdorferi*, *Nessieria menignitidis*, *Yersinia pestis*, *Campylobacter jejuni*, *Vibrio cholerae*, *V. harveyi*, *V. parahaemolyiticus*, *V. vulnificus*, *Mycobacterium tuberculosis*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *S. pyogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *C. difficile*, *Porphorymonas gingivalis*, *Fusobacterium nucleatum*, *Yersinia enterocolitica*, *Y. pestis*, *Camplyobacteri jejuni*, *Heamophilus influenza*, *Shewenella putrafaceins*, *Bacillus anthracis*. *Bacillus anthracis*, *Neiseria gonorrheae*, *Borrelia burgdorferi* and *Klebsiella pneumonia*.

In a still further preferred embodiment the microorganism is selected from the group consisting of *Vibrio harveyi*, *V. cholerae*, *V. parahaemolyticus*,

*V. anguillarum, V. alginolyticus, V. angustum S14, V. vulnificus, Yersinia enterocolitica, Y. pestis, Escherichia coli, Salmonella typhimurium, S. typhi, Camplyobacteri jejuni, Heamophilus influenza, Shewenella putrafaceins, Enterococcus faecalis, Bacillus anthracis, Helicobacter pylori, Mycobacterium tuberculosis, Streptococcus pneumonia, Klebsiella pneumonia, Neiseria gonorrheae*, and *Borrelia burgdorferi.*

In another preferred embodiment of the present invention the compound is selected from the group consisting of 2, 3, 24, 26, 30, 34, 56, 57, 60, 63, 64, 70, 72, 73, 74, 75, 78, 80, 85, 88, 91, 99, 102, 103, 105, 112, 113, 118, 124, 129, 139 as shown in Table 4.

The term "effective concentration" means a concentration sufficient to inhibit the particular virulence factor of interest. Such a concentration may be determined by simple experiments. The present inventors have found that typically virulence factors may be inhibited at concentrations of the compound insufficient to inhibit growth of the microorganism.

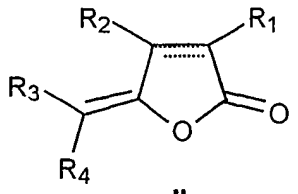
The compounds of formula II, III and IV act *in vitro* at concentrations that do not inhibit growth of the AI-2 monitor strains, as determined by either the bioluminescence or the metalloprotease assays described herein. These concentrations are normally in the range of about 0.1 to 100 μg/ml. It will be appreciated, however, the concentration required may depend on a number of factors including the microorganism, the furanone compound(s) used, the AI-2 system to be inhibited, and the formulation of the furanone.

In a third aspect, the present invention provides a method of treating an animal to decrease the severity of symptoms resulting from infection by bacteria with an AI-2 signalling system pathway, the method comprising administering to the animal an effective dose of a substance that capable of blocking the signalling cascade of the AI-2 signalling pathway.

The animal may be a human or a non-human.

The substance may be a furanone or related compound.

In a fourth aspect the present invention provides a method of treating an animal to decrease the severity of symptoms resulting from bacterial infection, the method comprising administering to the animal an effective dose of a composition comprising a compound of general formula II:




II

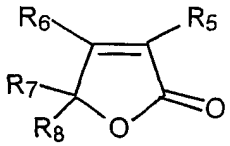
wherein $R_1$ and $R_2$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_3$ and $R_4$ are independently H, halogen, alkyl, aryl or arylalkyl, alkoxy, alkylsilyl or

$R_3$ or $R_4$ + $R_2$ can be a saturated or an unsaturated cycloalkane; and

"-------" represents a single bond or a double bond,

or a compound of general formula III:

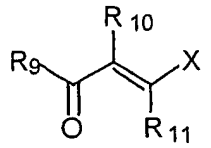



III

wherein $R_5$ and $R_6$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_7$ and $R_8$ are independently H, halogen, hydroxy, alkyl, haloalkyl, dihaloalkyl, trihaloalkyl, aryl, arylalkyl, a hydroxy compound or a derivative thereof or $R_7$ or $R_8$ + $R_6$ is saturated or unsaturated cycloalkane.

or a compound of general formula IV




IV

wherein $R_{10}$ and $R_{11}$ are independently H, halogen, carboxyl, ester, formyl, cyano, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

X is a halogen; and

$R_9$ is H, alkyl, alkenyl, alkynyl, alkene, alkyne, aryl, arylalkyl, whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic.

As will be recognised by those skilled in the art, the compounds of general formulas II, III and IV can exist as two isomers e and z. It is intended that the general formulas encompass both isomers either in the form of a mixture or separated isomers.

The term "aryl" as used herein refers to a moiety having one to about five rings, of which at least half are aromatic. Exemplary aryl groups include, without limitation, phenyl, naphthyl, biphenyl, terphenyl, 2-phenyl-naphthyl, anthryl, phenanthryl, and the like.

The term "alkyl" as used herein is taken to mean both straight chain alkyl groups such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tertiary butyl, and the like. Preferably the alkyl group is a lower alkyl of 1 to 6 carbon atoms. The alkyl group may optionally be substituted by one or more groups selected from alkyl, cycloalkyl, alkenyl, alkynyl, halo, haloalkyl, haloalkynyl, hydroxy, alkoxy, alkenyloxy, haloalkoxy, haloalkenyloxy, nitro, amino, nitroalkyl, nitroalkenyl, nitroalkynyl, nitroheterocyclyl, alkylamino, dialkylamino, alkenylamine, alkynylamino, acyl, alkenoyl, alkynoyl, acylamino, diacylamino, acyloxy, alkylsulfonyloxy, heterocyclyl, heterocycloxy, heterocyclamino, haloheterocyclyl, alkylsulfenyl, alkylcarbonyloxy, alkylthio, acylthio, phosphorus-containing groups such as phosphono and phosphinyl.

The term "alkoxy" as used herein denotes straight chain or branched alkyloxy, preferably $C_{1-10}$ alkoxy. Examples include methoxy, ethoxy, n-propoxy, isopropoxy and the different butoxy isomers.

The term "alkenyl" as used herein denotes groups formed from straight chain, branched or mono- or polycyclic alkenes and polyene. Substituents include mono- or poly-unsaturated alkyl or cycloalkyl groups as previously defined, preferably $C_{2-10}$ alkenyl. Examples of alkenyl include vinyl, allyl, 1-methylvinyl, butenyl, iso-butenyl, 3-methyl-2-butenyl, 1-pentenyl, cyclopentenyl, 1-methyl-cyclopentenyl, 1-hexenyl, 3-hexenyl, cyclohexenyl, 1-heptenyl, 3-heptenyl, 1-octenyl, cyclooctenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 1-decenyl, 3-decenyl, 1,3-butadienyl, 1-4,pentadienyl, 1,3-cyclopentadienyl, 1,3-hexadienyl, 1,4-hexadienyl, 1,3-cyclohexadienyl, 1,4-cyclohexadienyl, 1,3-cycloheptadienyl, 1,3,5-cycloheptatrienyl, or 1,3,5,7-cyclooctatetraenyl.

The term "halogen" as used herein denotes fluorine, chlorine, bromine or iodine, preferably bromine or fluorine.

The term "heteroatoms" as used herein denotes O, N or S.

The term "acyl" used either alone or in compound words such as "acyloxy", "acylthio", "acylamino" or diacylamino" denotes an aliphatic acyl group and an acyl group containing a heterocyclic ring which is referred to as heterocyclic acyl, preferably a $C_{1-10}$ alkanoyl. Examples of acyl include carbamoyl; straight chain or branched alkanoyl, such as formyl, acetyl, propanoyl, butanoyl, 2-methylpropanoyl, pentanoyl, 2,2-dimethylpropanoyl, hexanoyl, heptanoyl, octanoyl, nonanoyl, decanoyl; alkoxycarbonyl, such as methoxycarbonyl, ethoxycarbonyl, t-butoxycarbonyl, t-pentyloxycarbonyl or heptyloxycarbonyl; cycloalkanecarbonyl such as cyclopropanecarbonyl cyclobutanecarbonyl, cyclopentanecarbonyl or cyclohexanecarbonyl; alkanesulfonyl, such as methanesulfonyl or ethanesulfonyl; alkoxysulfonyl, such as methoxysulfonyl or ethoxysulfonyl; heterocycloalkanecarbonyl; heterocyclyoalkanoyl, such as pyrrolidinylacetyl, pyrrolidinylpropanoyl, pyrrolidinylbutanoyl, pyrrolidinylpentanoyl, pyrrolidinylhexanoyl or thiazolidinylacetyl; heterocyclylalkenoyl, such as heterocyclylpropenoyl, heterocyclylbutenoyl, heterocyclylpentenoyl or heterocyclylhexenoyl; or heterocyclylglyoxyloyl, such as, thiazolidinylglyoxyloyl or pyrrolidinylglyoxyloyl.

The term "fluorophilic" is used to indicate the highly attractive interactions between certain groups, such as highly fluorinated alkyl groups of C4-C10 chain length, have for perfluoroalkanes and perfluoroalkane polymers.

The term "cycloalkane" refers to a saturated hydrocarbon or unsaturated radical having one to three rings, and containing from three to about nine carbon atoms in the ring structure. Cycloalkyl moieties include, without limitation, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, 3-methylcyclohexyl, bicyclooctyl, norbornyl, and the like.

As will be recognised by those skilled in the art, the findings of the present inventors suggest a number of other useful applications. The method may be used in any environmental, sanitary, veterinary, or medical application where it is possible to affect the phenotype of a microorganism, particularly through inhibition of the AI-2 system and the present invention extends to those applications.

In any of the applications described herein, a particular AI-2 system may be targeted by use or selection of the compound or mixture of compounds. Similarly, a particular microorganism may be targeted by use or selection of the compound or mixture of compounds.

Examples of a pharmaceutical application of the method of the present invention are those in which the furanones and related compounds would be administered as an injection or as a tablet/pill to treat an infection, much in the same was as is now done for standard antibiotics. Clearly such a pharmaceutical applications would be useful in the treatment of human and non-human animals.

An application of the invention provides a topical dressing for burn patients comprising a compound of Formula II, III or IV or combinations of two or more thereof. In this regard they may be directly incorporated into bandages and the like. The compositions may also be formulated for other topical applications, for example, in application to wounds and the like.

In yet a further embodiment, the present invention provides a method for treatment and prevention of dental caries comprising administering composition comprising an effective amount of a compound of Formula II, III or IV or combinations of two or more thereof.

The invention also provides a method for the treatment or amelioration of dental disease or gingivitis comprising administration of a compound of Formula II, III or IV or combinations of two or more thereof.

In another aspect, the present invention provides a method for the treatment of acne comprising administering a composition a compound of Formula II, III or IV or combinations of two or more thereof.

The present invention provides a method of regulating biofilm development comprising administering a composition comprising a compound according to according to Formula II, III or IV or combinations of two or more thereof.

Also provided is a method for prevention of biofilm matrix polymer development comprising administering a composition comprising comprising a compound according to according to Formula II, III or IV or combinations of two or more thereof.

In yet a further embodiment, the present invention provides a method of biofilm dispersion comprising administering a composition comprising a compound according to Formula II, III or IV or combinations of two or more thereof.

The invention further provides a method of prevention of biofilm dispersion comprising administering an effective amount of a composition comprising a compound of Formula II, III or IV or combinations of two or more thereof.

Further applications include, but are not limited to, inhibition of growth of microbial pathogens in environmental situations, reduction or prevention of microbial colonisation of medical media including washing solutions, ointments and the like, inhibition of microbial attachment to surfaces and subsequent biofilm formation, as active ingredients in antiseptics and disinfectants.

As will be recognised by those skilled in the art the compounds of formulae II and III and IV can be usefully incorporated in a varied range of compositions. For example the compounds can be incorporated in a range of personal care products including body care and oral care such as deodorants, soaps, shampoos, dentifrices, mouthwashes etc. The manufacture of such compositions is well known in the art and the compounds or mixtures thereof can be simply included in these compositions in admixture.

The present invention also provides for a cleaning composition for cleaning surfaces, for example hard surfaces, woven or unwoven surfaces. Examples of surfaces which may be cleaned and/or cleaning compositions of the invention include toilet bowls, bath tubs, drains. high chairs, countertops (such as those exposed to meats ,vegetables), meat processing rooms, butcher shops, airducts, airconditioners, carpets, paper or woven product treatment, diapers and healthy air machines.

The cleaning product may be laundry liquid or powder or the like comprising a compound of Formula II, III or IV.

The cleaning product may be in the form of a toilet drop-in for prevention and removal of soil and under rim cleaner for toilets.

The method of the present invention will also find application in preventing or inhibition biofilm formation. In another embodiment the compositions will find application as washing solutions, particularly in contact lens cleaning compositions. Thus contact lenses can be cleaned and disinfected by administering a composition comprising an effective amount a compound of Formula II, III or IV or combinations of two or more thereof.

Also provided is a method of treating/(disinfecting and cleaning) medical indwelling devices comprising administering a composition comprising an effective amount of a compound of Formula II, III or IV or combinations of two or more thereof.

These devices may advantageously include, for example, any indwelling device, for example catheters, orthopedic devices and implants.

The invention provides for a microbial disinfectant composition comprising an effective amount of a composition according to Formula II, III or IV or combinations thereof.

The invention provides a method of preventing biofilm formation by contacting the microorganism with a compound of Formula II, III or IV, or combinations thereof.

In still another embodiment the invention provides a method of preventing fouling comprising administering a composition a compound of Formula II, III or IV or combinations of two or more thereof. The fouling may be in a system selected from injection wells for oil recovery, cooling towers, water purification systems, porous media (soil, sand), marine environments and hospital or automotive airconditioning systems.

The invention, further provides a method for the dispersal of

environmental concentrations of bacteria comprising administering a compound of Formula II, III or IV or combinations of two or more thereof.

In yet another application, the invention provides a disinfectant coating comprising a compound of Formula II, III or IV or combinations of two or more thereof copolymerized to a polymer or blended with a polymer.

The coating may be applied to drains, shower curtains, grout, toilets, flooring or other domestic or industrial surface.

The compound used in the method of the invention may be incorporated into a polymer either by blending the compound with the polymer or incorporating the compound(s) as a monomer in polymer.

The compound of the invention may be incorporated into personal care products and skin care products. The compounds of the invention may be used in the preparation of epidermal bandages and lotions. In an alternative embodiment, the compounds of the invention may be incorporated into, for example, aftershaves or lotions.

Other application of the method of the invention include, for example, use with oysters where the furanones and related compounds could be used to induce the VBNC response in *Vibrio* contaminants, which would essentially make the *Vibrio* cells dormant and not capable of expression of virulence. This could be achieved by holding the oysters in a recirculating seawater bath where the furanones and related compounds are added to the water.

Further, as it appears that *V. haveyi* regulates its virulence factor production through the AI-2 system, the incorporation of the furanones and related compounds into prawn feeds for the prevention of disease in the prawns may be another beneficial application.

The present invention further provides a compositions comprising a compound of Formula II, III or IV, or combinations of two or more thereof suitable for the inhibition of virulence in a microorganism.

The composition may include a biocide and/or antibiotic. The compositions may include a surfactant.

It is intended that such applications are within the scope of the present invention.

DETAILED DESCRIPTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting examples.

**FIGURE LEGENDS**

FIG. 1. Effect of growth phase on the production of substances able to induce luminescence in *V. harveyi* BB170. *V. vulnificus* C7184(T) was grown with aeration in 2M medium at 37°C and optical density (absorbance at 610 nm) was determined (squares). Cell-free supernatants were prepared at various times and assayed for their ability to induce luminescence in the *V. harveyi* reporter strain (bars). The activity of supernatants is presented as the percentage of activity obtained when *V. harveyi* BB152 cell-free spent supernatant is added to the reporter strain. Results presented here are representative of results obtained in at least 3 independent experiments.

FIG. 2. Construction of the *smcR* mutant, *Vibrio vulnificus* DM7. The following steps were performed to make a null mutation in the *smcR* gene. The *sacB* gene from pCVD442 was excised as a *Eco*RV-*Pst*I fragment and was inserted into the *Eco*RV-*Pst*I site of pLG401 at the 5' position relative to the promoterless *gfp* gene to make the suicide delivery plasmid pMacSB (Step 1). The *smcR* gene was PCR amplified, ligated into pUC19 and transferred into the *Eco*RI-*Hind*III site of pBluescriptII SK to make the plasmid pSmcR8.18. The streptomycin resistance cassette from pCAM140 was excised as a *Bam*HI fragment and inserted into the *Bgl*II site of pSmcR to generate the plasmid pSmcR.SM (Step 2). The disrupted *smcR* gene from pSmcR.SM was amplified by PCR and ligated into the *Eco*RVsite of pMacSB to make pMacSmcRK (Step 3). The plasmid pMacSmcRK was conjugated into *V. vulnificus* C7184 (O) and colonies that were streptomycin resistant and chloramphenicol sensitive were isolated (Step 4). Confirmation of the mutant was made by Southern hybridization and by PCR; one mutant, *V. vulnificus* DM7, was chosen for further characterization. The orientation of the *smcR* gene with its streptomycin insertion as well as the orientation of the genes 5' and 3' of *smcR* is shown. Arrows above the genes indicate the direction of

transcription for *smcR*, *lpd* (encoding dihydrolipoamide dehydrogenase in *E. coli*), *hpt* (encoding hypoxanthine ribosyltransferase in *E. coli*) and the gene for streptomycin resistance, Sm/Sp. *mob*, $Cm^r$, *ori*, *gfp*, and *sacB* refer to genes that code for plasmid mobilization, choramphenicol resistance, green fluorescent protein, and sucrose sensitivity respectively.

FIG. 3. Exoprotease activity of *V. vulnificus* C7814O and DM7 (*smcR*::Sm). Cultures of *V. vulnificus* C7814O (□,■) and DM7 (○,●) were grown in LB at 37°C with shaking at 200 rpm on a rotary shaker. At various time points, aliquots were removed and cell-free supernatants prepared by centrifugation (10,000 χ g; 10 min) followed by filtration of the supernatant through 0.2 μm filters. Exoprotease activity (closed symbols) was assayed by degradation of HPA (A), azocasein (B) and elastin-Congo red (C) at 37°C. Results are presented as the exoprotease activity per cell and are representative of at least three independent experiments.

FIG. 4. SmcR affects starvation survival and starvation-induced maintenance of culturability (SIMC) at low temperature. *V. vulnificus* C7184(O) (filled symbols A and B) and DM7 (*smcR*::Sm) (open symbols A and B) were grown to mid-exponential phase in LB with 20 g $l^{-1}$ NaCl, the cells collected by centrifugation (10,000 χ g; 10 min), washed in 2M lacking glucose (2M-C) and resuspended in 2M-C. Cultures were held statically at 24 °C (A) (☒, ⊞) or were allowed to starve (B) for 0 (B,) or 4 hours (🏞, ★) before shifting to 4°C. Data shown in C represents C7184 starved in the presence (open symbols) or absence (closed symbols) of C2. Determination of CFUs were performed on DVNSS agar plates. Data are presented as percentages of the initial count (1.1 - 2.9 x $10^5$ CFU $ml^{-1}$) and are representative of three independent experiments. Error bars represent the 95% confidence interval.

**Materials and methods**

**Bacterial strains and growth conditions**

The plasmids and bacterial strains used in this study and their genotypes are listed in Table 1. *V. vulnificus* C7184(O) is a clinical isolate obtained from the drainage of a hand wound (46). *V. vulnificus* C7184(T) is a spontaneously derived non-encapsulated and thus non-virulent mutant of the opaque strain C7184 (58). The designations (T) and (O) refer to translucent and opaque colony morphologies respectively, on agar plates which is related to capsular polysaccharide production. The *V. harveyi* strains were a gift

from Bonnie Bassler. *V. harveyi* BB170 (3) (sensor $1^-$, sensor $2^+$) has a null mutation in the gene for sensor 1 and thus is a reporter strain for autoinducer 2 (AI-2). *V. harveyi* BB152 (3) (autoinducer 1-, autoinducer 2+) has a null mutation in the gene for autoinducer 1 synthase (AI-1) and produces only AI-2, and thus serves as a positive control for assays of AI-2 production.

Strains of *E. coli* and *V. vulnificus* were stored at -70°C in Luria-Bertani (LB) (38) broth (10 g tryptone (Oxoid), 5 g yeast extract (Oxoid) and 10 g NaCl per liter of distilled water) containing 15% (vol/vol) glycerol (Research Organics), while other *Vibrios* were maintained in LB with 20 g NaCl per liter. Prior to each experiment, cells were inoculated from frozen stocks into LB broth overnight and then plated onto LB agar (LB with 15 g agar (Research Organics) per liter distilled water) to check for purity. Where specified, glucose was added from a filter-sterilized 20% stock to a final concentration of 0.5% for LB and 0.4% for 2M minimal medium (48). Antibiotics were used at the following concentrations ($\mu g\ ml^{-1}$) ampicillin 50, streptomycin 200, chloramphenicol 34 and colistin 100.

For starvation and cold incubation experiments, cells were grown in LB with 20 g $l^{-1}$ NaCl overnight at room temperature, transferred to fresh medium at a dilution of 1:50 and grown overnight. Cells were then transferred to fresh medium again at a dilution of 1:100. The cells were grown to mid-exponential phase (optical density at $OD_{610}$ nm = 0.2; 4.0 ■ $10^8$ CFU $ml^{-1}$) and harvested by centrifugation (10,000 ■ g, 10 min, 24°C, Beckman Avanti J-25 I centrifuge, JA 25.50 rotor) and washed twice in 0.5 ■ NSS (48) (0.5 ■ NSS consisted of: NaCl, 8.8 g; $Na_2SO_4$, 0.735 g; $NaHCO_3$, 0.04 g; KCl, 0.125 g; KBr, 0.02 g; $MgCl_2 \cdot 6H_2O$, 0.935 g; $CaCl_2 \cdot 2H_2O$, 0.205 g; $SrCl_2 \cdot 6H_2O$, 0.004 g; $H_3BO_3$, 0.004 g; double distilled $H_2O$, 1000 ml; the pH was adjusted to 6.5 prior to autoclaving with KOH so that the pH after autoclaving is 7.5). Cells were resuspended in 2M medium lacking glucose (2M-C) (48) at 1:100 dilution. Sub-samples of these cell suspensions were left at room temperature or starved for 0 or 4 hours at 24°C and then shifted to 4°C. All samples were incubated in the dark. The starvation time was calculated from the start of the first wash. For the determination of colony forming units, cells were serially diluted in 0.5 ■ NSS and plated onto DVNSS agar plates (1.0 g peptone (Oxoid), 0.5 g yeast extract (Oxoid), 0.5 g glucose, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $Na_2HPO_4$ per liter of 0.5 ■ NSS) (48) which were incubated overnight at 37°C. Colony forming units were determined by the drop plate

method (19). Agar (Research Organics) was added to a concentration of 1.5% for solid media. General chemicals were purchased from Sigma (Sigma Chemical Co., St. Louis, MO).

**Preparation of cell-free supernatants**

For the generation of cell-free supernatants, *V. harveyi* strains were grown in autoinducer bioassay (AB) medium (15) overnight at 30°C with shaking at 200 rpm. AB medium consists of 0.3 M NaCl, 0.05 M $MgSO_4$, and 0.2% vitamin-free casamino acids (Difco), adjusted to pH 7.5 with KOH. The medium was sterilized, allowed to cool, and 10 ml of sterile 1 M potassium (pH 7.0), 10 ml of 0.1 M L-arginine, 20 ml of glycerol, 1 ml of 10 $\mu$g $ml^{-1}$ of riboflavin, and 1 ml of 1 mg $ml^{-1}$ of thiamine was added per liter. *V. vulnificus* strains were grown in LB, LB + 0.5% glucose or 2M (48) medium at 24°C or 37°C overnight. The following morning, fresh medium was inoculated at a 1:100 dilution and the cultures were grown to late stationary phase. Growth was followed spectrophotometrically with a Pharmacia Biotech model Novaspec II spectrophotometer and aliquots removed at various time points. Supernatants for the *V. harveyi* bioluminescence bioassay were prepared by the removal of cells by centrifugation of cultures (8,000 ■ g, 24°C for 10 min in a Beckman Avanti J-25 I centrifuge, JA 25.50 rotor) followed by filtration of the supernatant through 0.2 $\mu$m pore size filters (Supor Acrodisc, Pall-Gelman Laboratories, Ann Arbor, MI). Supernatants were frozen at -20°C until used.

In experiments performed with the addition of signal antagonists, the furanone compound 2 was added to cultures at a concentration of 2 $\mu$g $ml^{-1}$ at time 0 for the VBNC assay and 10 $\mu$g $ml^{-1}$ during exponential phase ($OD_{610}$ nm = 0.4) for the signal production experiment. The cell-free supernatants collected for the signal production experiment were also the same ones tested in the HPA protease assay for inhibition of protease production by furanones. Stock solutions of furanone compounds were prepared in EtOH at a concentration of 20 mg $ml^{-1}$. EtOH was added to control samples to account for the solvent volume added and growth was followed spectrophometrically to ensure the concentrations of compound used were not growth inhibitory.

***V. harveyi* bioassay for the detection of AI-2 activity**

Cell-free supernatants obtained from strains of *V. vulnificus* were tested for their ability to induce luminescence in the *V. harveyi* AI-2 reporter strain BB170. The effect of growth phase and growth medium on the production of AI-2-like signals in different strains was examined. *V. harveyi* BB152 (AI-1-, AI-2+) supernatant was used as a positive control and *E. coli* DH5α supernatant or sterile media as a negative control. This strain of *E. coli* has previously been shown to harbor a mutation in the AI-2 synthase gene, *ygaG*, which results in a truncated protein with no AI-2 activity (62). The *V. harveyi* bioassay was performed as described previously (61). Briefly, 10 μl of the above-described supernatants were added to wells of 96-well microtiter plates. The *V. harveyi* reporter strain BB170 was grown for 16 hours at 30°C with shaking in AB medium. Cells were diluted 1:5,000 into 30°C prewarmed AB medium and 90 μl of the diluted suspension was added to wells containing supernatant. The microtiter plates were incubated at 30°C with shaking at 175 rpm. Hourly determinations of the total luminescence were quantified using the chemiluminescent setting on a Wallac (Gaithersburg, MD) model 1450 Microbeta Plus liquid scintillation counter. The *V. harveyi* cell density was monitored by the use of a microplate reader (Bio-Rad, Hercules, CA). Activity is reported as the percentage of activity obtained from *V. harveyi* BB152 cell-free supernatant. While the absolute values of luminescence varied considerably between experiments, the pattern of results obtained was reproducible.

**Tests on activity of furanones and related compounds**

The experiment was set up and run as described by Bassler *et al.* (2). Two strains of *Vibrio harveyi* were used. *V. harveyi* BB170, which is HBHL sensor negative (luxN-) serves as the monitor strain, while *V. harveyi* BB152, which is deficient in HBHL production serves as the AI-2 producing strain. The monitor strain, BB170, was diluted 1:5000 into fresh LB20 medium from an overnight culture into the wells of a sterile 96 well microplate for the Wallac MircroBeta Plus Bioluminescence counter. Sterile filtered supernatant from BB152 was collected from an overnight culture (grown in AB medium at 30°C) and added to strain BB170 at 10% to serve as the positive control. BB170 cells with no addition served as the negative control and also served as a measure for the natural induction since the BB170 strain will eventually bioluminesce on its own. Compounds were added to wells

containing BB170 (without the BB152 supernatant) at various concentrations. The plate was incubated at 30°C with shaking. At different times, the plate was removed from the incubator and the bioluminescence determined. To quantify the amount of induction of the BB170, readings were taken at a time when the positive control, containing the BB152 supernatant, was highest and the negative control, or natural luminescence, was lowest. Induction was then compared to the positive control values, which were arbitrarily set at 100%. Inhibition of luminescence was determined by continuing to monitor luminescence until the bioluminescence of the negative control was equal to or greater than the positive control. The inhibitory level was calculated by setting the negative control, or natural induction, at 100%.

**Molecular biology techniques**

Genomic DNA was isolated by the method of Tillett and Neilan (67). Plasmid DNA was isolated by the alkaline lysis method using the QIAFILTER Maxi Plasmid kit (QIAGEN, Pty. Ltd., Clifton Hills, Vic., Australia) for large scale purification or by the use of Wizard® *Plus* Minipreps (Promega Corp., Madison, Wisconsin, USA) for small scale purification.

A probe for the identification of *smcR* containing the streptomycin insert was generated by PCR with the primers SmcR (5'. GCGTAGTGTATCATTCCGC) and LuxR3r (GGTCACACACTTCACCACGC). The PCR product of 700-800 bp was gel purified, electroeluted and used as a template for digoxigenin (DIG) labeling (Boehringer Mannheim, Indianapolis, IN) by PCR. For Southern hybridizations, chromosomal DNA was digested with *Sal*I and electrophoresed on a 0.7% agarose gel in Tris-borate buffer. The digested DNA was transferred to Hybond N+ membranes (Amersham Life Science, Buckinghamshire, England) using a BioRad vacuum blotter (Model 785, BioRad, Richmond, CA). Membranes were hybridized at 50°C for 18 hours in a high SDS buffer (7). Pre-hybridization and hybridization washes were performed at 50°C; post-hybridization washes were one rinse with 2 ■ SSC containing 0.1% SDS at room temperature and two rinses with 0.1 ■ SSC containing 0.1% SDS. Detection was carried out according to manufacturer recommendations using an anti-DIG antibody conjugated to alkaline phosphatase and the chemiluminescent substrate CSPD® (Boehringer Mannheim), and was visualized by autoradiography using X-Omat AR film (Eastman Kodak Co., Rochester, NY).

PCR amplifications were performed in an automatic thermal cycler (Hybaid PCR Sprint, Hybaid Limited, Middlesex, UK) using standard conditions. Restriction enzymes, molecular weight markers, shrimp alkaline phosphatase, ligase, *Pwo* and T4 DNA ligase were purchased from Boehringer Mannheim.

**Generation of *V. vulnificus smcR* null mutants**

A 720 bp fragment from *V. vulnificus* C7184(O) was amplified with *Pwo* polymerase (Boehringer Mannheim) using the LuxR2f (5' GCACAATTACACTCATCAGTG) and LuxR2r (5' GTTCACGGTTGTAGATGCATAGC) primers, which were derived from the sequence of *V. vulnificus smcR* (stationary phase induced maintenance of culturability regulator) (34) (accession no. AF204737). The PCR product was gel purified by excising the band from a 0.7% agarose gel in Tris-borate buffer and then electroeluting the fragment from the gel slice. The purified product was blunt-end cloned into the *Sma*I site of pUC19 (74). Positive clones were confirmed by sequencing and one clone, pSmcR8, was selected for subsequent work. The *smcR* gene was excised from pUC19 using the restriction endonucleases *Eco*RI and *Hind*III and the fragment was cloned into pBluescript® II SK (Stratagene, La Jolle, CA) which had been prepared by digestion using *Eco*RI and *Hind*III. This clone was designated pSmcR8.18. The *smcR* gene on plasmid pSmcR8.18 was disrupted by insertion of a selectable marker, streptomycin, into the *Bgl*II site 183 bp downstream from the ATG codon. The 2.0 kb streptomycin resistance cassette was removed from pCAM140 (70) with *Bam*HI and gel purified as described above. pSmcR8.18 was digested with *Bgl*II, dephosphorylated with shrimp alkaline phosphatase (Boehringer Mannheim) and the purified 2.0 kb streptomycin gene cassette was ligated into the *Bgl*II site. Positive clones containing the *smcR* gene with the streptomycin disruption were selected on LB plates supplemented with 200 $\mu$g ml$^{-1}$ streptomycin and were confirmed by sequencing. The plasmid carrying the disrupted *smcR* gene has been designated pSmcR.Sm.

The present inventors constructed a vector for delivery and homologous recombination of the disrupted *smcR* that would replicate in *V. vulnificus*, but that carries a counter-selectable marker, the *sacB* gene. The expression of *sacB* causes cell lysis when cells are grown in the presence of

sucrose under low osmolarity and thus enables us to select for cells that have either lost the plasmid or in which a double cross-over event had occurred, leaving the disrupted *smcR* gene on the chromosome. This plasmid was constructed by inserting the *sacB* gene as a *Pst*I-*Eco*RV fragment from pCVD442 (8) into the *Pst*I-*Eco*RV site of pLG401 (constructed by Lynn Gilson, University of Hawaii) and was denoted pMacSB. The disrupted *smcR* gene was then ligated into the *Eco*RV site of pMacSB.

This plasmid, pMacSmcRK, was then conjugated into *V. vulnificus* C7184(O) in the following manner. *V. vulnificus* was grown in brain heart infusion (BHI) broth (Difco, Detroit, Michigan, USA) containing 2% NaCl and *E. coli* BW20767 in LB with antibiotics for 16 - 18 hours at 37°C with shaking at 200 rpm. *E. coli* was washed once in prewarmed LB and allowed to stand 30 min prior to conjugation. *V. vulnificus* was heat shocked at 45°C for 15 min, washed in LB and a 1.5 ml cell pellet was resuspended in 50 $\mu$l of prewarmed LB. Cell suspensions were mixed (50 $\mu$l donor: 150 $\mu$l recipient) on a filter (GS Millipore; 0.22 $\mu$m pore size) placed on the surface of an LB agar plate at 37°C for 16 hr. Following incubation, the filters were resuspended in 2 ml of 10 mM $MgSO_4$ and aliquots plated onto selective media. Transconjugants, carrying pMacSmcRK were selected on LB agar supplemented with streptomycin (200 $\mu g\ ml^{-1}$) and colistin (100 $\mu g\ ml^{-1}$). To select for double crossover events, cells were grown at 30°C in LB supplemented with 0.05% NaCl (*V. vulnificus* would not grow without NaCl) and 6% sucrose. Cultures that grew under these conditions were then plated onto LB supplemented with streptomycin. The streptomycin resistant colonies were tested for sensitivity to the antibiotic carried on the plasmid, chloramphenicol, which indicated that the clones had the disrupted *smcR* gene, but did not carry the entire plasmid as an insert.

The disruption of the *smcR* gene in one of the clones, *V. vulnificus* DM7, was confirmed by Southern hybridization using a probe that binds to the 5' half of the gene (see above). The *V. vulnificus* DM7 mutant had a chromosomal band shift when compared to the wild-type. Further confirmation was obtained by PCR.

**Electron microscopy**

For viewing by electron microscopy, *V. vulnificus* cells were fixed in 1% glutaraldehyde. One drop of the cell suspension was placed onto a 200

mesh carbon-coated grid and left for 1-2 min. The drop was removed with filter paper and the grid washed 3 times with $dH_2O$ and then negatively stained with 2% uranyl acetate in water. The negative stains were examined and photographed with a Hitachi H-7000 transmission electron microscope operating at 75 kV.

**Biofilm assays**

The biofilm assays were performed in 96 well microtiter plates. Log phase cells were added to wells at an $OD_{610}$ nm of 0.1 in 100 $\mu$l LB. Biofilm formation was allowed to proceed overnight, wells were washed twice with PBS to remove loosely attached cells and the attached cells stained with 100 $\mu$l of a 1% crystal violet solution for 15 min. After staining, the crystal violet solution was removed and the well washed three times with PBS (twice with 300 $\mu$l and once with 100 $\mu$l). The crystal violet that remained bound to cells was then extracted with 200 $\mu$l of 95% ethanol for at least 5 min. The ethanol solution was transferred to a cuvette and the absorbance at 540 nm was determined spectrophotometrically.

**Exoenzyme assays**

For studies on the regulation of exoprotease activity, cell-free supernatants were prepared as described above for bioluminescent assays. Samples were collected at various time points throughout the growth of the cultures in LB, cell-free supernatants were prepared and frozen at -20°C until analysis. For the determination of exoenzyme activity, several substrates were utilized. The Hyde powder azure (HPA) assay was performed by the addition of 1 mg $ml^{-1}$ HPA to cell-free supernatants that were incubated at 37°C for 4 hours. The samples were centrifuged at 8,000 g for 5 min to pellet cells and undegraded substrate and the supernatant collected by filtration through a 0.2 $\mu$m disposable filter (Supor Acrodisc, Pall Gelman Laboratories, Ann Arbor, MI). Protease activity per cell of the collected supernatant was determined as the absorbance at 595 nm divided by the $OD_{610}$ nm of the culture. In the experiment with the addition of the signal antagonist (see above), furanone compound 2, the compound was added during exponential phase ($OD_{610}$ nm = 0.4) and the culture grown in the presence of the furanone until early stationary phase at which time cell-free supernatants were collected.

For the azocasein assay, samples were prepared as described for the HPA assay and 100 μl of supernatant was added to 900 μl of azocasein solution (1% in 0.1 M Tris pH 8.0). The solution was incubated for 4 hours at 37°C and the reaction stopped with the addition of 2 ml of 10% trichloroacetic acid. The solution was centrifuged at 10,000 g for 5 min to remove precipitated protein and the supernatant collected and the absorbance at 440 nm assayed. The activity per cell is calculated by the division of the absorbance at 440 nm by the OD at 610 nm.

The elastase assay was performed with supernatants collected as described above and 50 μl added to 1 ml of buffer (0.1 M Tris pH 7.2; 1 mM $CaCl_2$) containing 20 mg elastin-Congo red (Sigma Chemical Co., St. Louis, MO). The mixture was incubated at 37°C for 6 hours and insoluble protein removed by centrifugation at 10,000 g for 5 min. Elastase activity per cell is determined as the absorbance at 495 nm divided by the OD at 610 nm.

Alkaline phosphatase assays were performed in 96 well microtiter plates. Cell-free supernatants collected throughout the growth of the culture were prepared as described above. The alkaline phosphatase substrate was prepared according the manufacturers instructions by adding one tablet of the Sigma 104 Phosphatase substrate tablet to 8 ml of diethanolamine buffer. Fifty μl of the reagent was added to 100 μl of bacterial supernatant in 96 well microtiter plates. The reaction was allowed to proceed at 37°C for 4 hours at which time the absorbance was read at 450 nm.

**Results**

**Production and regulation of AI-2 activity in *V. vulnificus***

***V. vulnificus* produces a substance that induces luminescence in *V. harveyi***

Numerous *Vibrio* species have been shown to possess signalling systems that can activate luminescence in *V. harveyi* bioassays. The similarities between the metalloproteases of *V. cholerae* and *V. vulnificus* and the observation that the *V. cholerae* HA/P is regulated by such a system, prompted us to investigate the occurrence of signalling system 2 in *V. vulnificus*. The addition of cell-free supernatants to the *V. harveyi* BB170 reporter strain was used to demonstrate the production of a signalling molecule by *V. vulnificus*. Both *V. vulnificus* C7814(T) and UTHS-1(O) induced bioluminescence in *V. harveyi* (data not shown). *V. vulnificus* cells were grown in LB with aeration at 37°C and supernatants were collected in

late exponential phase ($OD_{610}$ nm = 0.796). Cell-free supernatants were prepared and added to BB170 cells at a concentration of 10%. These supernatant preparations were able to induce the reporter strain to at least 100% activity as determined by the addition of supernatant from the *V. harveyi* AI-2 positive strain, BB152. Specifically, the *V. vulnificus* strain C7184 induced 215% and UTHS-1 induced 350% of the BB152 activity. These results indicate that *V. vulnificus* produces a compound that induces luminescence via the signal system 2 pathway and that at least two strains possess this ability.

**Effect of growth medium and temperature on the production of signal molecules in *V. vulnificus***

It has been shown that glucose regulates the production of signal molecules in some bacterial strains. For example, *S. typhimurium* and some strains of *E. coli* do not produce AI-2 activity without the addition of glucose to LB (62). To determine the effect of growth medium and temperature on the production of signal molecules in *V. vulnificus*, cells were grown in either LB, LB + 0.5% glucose or the minimal medium, 2M, at either room temperature or at 37°C. The results of a typical experiment are shown in Table 2. Cell-free supernatants were collected from C7814(T) and added to the *V. harveyi* reporter strain BB170 at a final concentration of 10% (vol/vol). Supernatant collected from *V. harveyi* strain BB152 ($AI\text{-}1^-$, $AI\text{-}2^+$) grown in AB media served as a positive control and sterile media as negative control. *V. vulnificus* C7184(T) or UTHS-1(O) (data not shown) grown in either LB or 2M medium can produce a signal which induces luminescence in the reporter strain (Table 2), and the production of this factor is not temperature-dependent as production occurred at both room temperature (data not shown) and 37°C. However, supernatants collected from cells grown in LB with the addition of 0.5% glucose do not induce luminescence of *V. harveyi* (Table 2). Supernatants collected from DM7 (*smcR*::Sm) were also able to induce luminescence in *V. harveyi* to similar levels as the wild type (data not shown), indicating that *smcR* is not required for signal production.

**Production of signal molecules is growth phase dependent in *V. vulnificus***

To investigate the kinetics of signal production in *V. vulnificus*, cells were grown at 37°C in 2M medium with aeration and samples were removed

throughout the growth curve for preparation of cell-free supernatants. A typical experiment is shown in Fig. 1 and the same trend was observed with cells grown in LB (data not shown). The production of AI-2 activity increased until late exponential to early stationary phase after which the activity of cell-free supernatants decreased. Often, the 24 hour samples had reduced or no ability to induce the *V. harveyi* reporter strain. These results indicate that production of signalling activity is growth phase-dependent.

Maximal signal production in *V. vulnificus* occurs as cells enter the stationary phase of growth. This led to speculation that nutrient and/or energy starvation may be involved in the induction of autoinducer production, as starvation is one of the cues that induces many stationary phase genes. In order to determine if the production of AI-2-like activity in *V. vulnificus* is induced under conditions of nutrient starvation, cells of C7184(T) or UTHS-1(O) were grown to mid-exponential phase in LB containing 20 g $l^{-1}$ NaCl and collected by centrifugation at 8,000 ■ g for 10 min at 24°C. Cells were washed once and resuspended in 0.5 ■ NSS. Supernatant samples were taken every hour and tested for AI-2-like activity (data not shown). Cell-free supernatants taken from cells immediately after the shift to starvation conditions (time = 0) induced luminescence in the *V. harveyi* reporter strain 0.4%. Induction of luminescence increased to 849% for supernatants taken from cells starved for 4 hr at which time their ability to induce luminescence began to decrease and by 9 hr of starvation had dropped to 245%. These data indicate that starvation conditions are able to stimulate the production of AI-2-like activity in *V. vulnificus*. The production of the luminescence inducing factors peaks between 4 - 6 hours of nutrient starvation and thereafter begins to decline. The cell numbers of the cultures did not increase during this time. These results indicate that cells that have been shifted from exponential growth to starvation conditions are able to produce signal molecules.

**Addition of signal antagonists do not inhibit signal production**

The marine red alga *Delisea pulchra* has been shown to produce a range of halogenated furanones that specifically inhibit signalling phenotypes regulated by the AHL systems in bacterial species. The furanone compound 2 (C2) was added to growing cultures of *V. vulnificus* C7184(T) in order to assess whether signal transduction is required for the production of

autoinducer activity. The response of *V. harveyi* BB170 to supernatants collected from *V. vulnificus* during growth with C2 is shown in Table 2. The addition of C2 did not inhibit signal production by this organism.

**Affect of furanones and related compounds on bioluminescence**

The results of experiments on the affect of a number of furanones and related compounds on bioluminescence are set out in Table 5. A number of these compounds were shown to be very effective in this assay. It has been previously shown that toxin production in V. harveyi is regulated by the quorum sensing systems and that toxin production correlates with bioluminescence.

**Characteristics of the *V. vulnificus* signalling molecule**

Attempts to extract the signal molecule from cell-free supernatant with dichloromethane (DCM) or ethyl acetate proved unsuccessful. While it was possible to extract some activity into the solvent phase, the majority of the activity always remained in the aqueous phase, which suggests the factor is a polar compound (data not shown). To investigate the heat lability of the signal produced by *V. vulnificus*, cell-free supernatants were prepared from cultures of C7184(T) grown in 2M medium to early stationary phase ($OD_{610}$ nm = 0.7). The autoinducer activity of cell-free supernatants heated to 80°C for 10 min was reduced by 52.7% while heat treatment at 100°C for 10 min abolished activity (Table 2). These results indicate that the AI-2 stimulating factor detected here is a heat labile compound.

***V. vulnificus* possesses the AI-2 synthase gene, *luxS***

The discovery of the signal synthase gene required for the production of AI-2 (62) in *V. harveyi*, *E. coli* and *S. typhimurium*, the observation that numerous bacterial strains possess highly conserved *luxS* homologues, and the demonstration of AI-2 activity in *V. vulnificus*, prompted the search for a *luxS* homologue in *V. vulnificus*. The *luxS* in *V. vulnificus* was identified by PCR amplification of an approximately 320 base pair fragment genomic DNA from *V. vulnificus* C7184(O) using degenerate primers (LuxS.F 5' cat (c/t)tg t(a/t)(c/t) gct ggc ttt atg and LuxSR 5' (a/c)(c/t)t ct(c/g) gca g(c/t)g cca att c) based on the alignment of *luxS* sequences found in the GenBank database. The amplified product was cloned and sequenced and it was determined that

the cloned fragment shared >80%, 79%, and 68% nucleotide identities to the *luxS* gene from *V. harveyi*, an unidentified open reading frame in the *V. cholerae* genome database, and the *ygaG* gene of *E. coli* (data not shown). The amplified region lies within the open reading frame that encodes the LuxS protein. Based on the high degree of nucleotide identity and the presence of AI-2 activity in the supernatants of *V. vulnificus*, the present inventors believe that they have identified a putative *luxS* gene in *V. vulnificus*.

**Characterization of a mutant in *V. vulnificus* of the *luxR* transcriptional regulator homologue**

In *V. harveyi,* the LuxR regulatory protein is required for the expression of the *lux* operon. The present inventors have previously reported the cloning and sequencing of a homologue of the *V. harveyi luxR* gene from *V. vulnificus, smcR* (stationary phase induced maintenance of culturability regulator (34). A potential *rho*-independent terminator lies nineteen nucleotides downstream of the *smcR* stop codon, and the *smcR* coding region is followed by a divergently transcribed homologue of *lpd* (dihydrolipoamide dehydrogenase) (Fig. 2). In an attempt to determine the role of the putative SmcR regulator in the signalling pathway, the present inventors generated a null mutation in the *smcR* gene (Fig. 2). Interruption of the *smcR* gene was achieved by the insertion of a selectable marker, streptomycin, 183 bp downstream from the ATG codon by homologous recombination. This disruption was confirmed in one of the clones, *V. vulnificus* DM7, by Southern hybridization using a probe that binds to the 5' half of the gene (see above). The *V. vulnificus* DM7 mutant had a chromosomal band shift when compared to the wild type band. Further confirmation was obtained by PCR.

**Capsule production by *V. vulnificus* DM7**

In *V. parahaemolyticus*, mutation of the *V. harveyi luxR* homologue, *opaR*, results in loss of capsule production (32). *V. vulnificus* produces an extracellular polysaccharide capsule that is regulated by growth phase and environmental conditions (73), and in addition, produces translucent mutants that synthesize reduced levels of capsular material (58). The *smcR* mutation was thus evaluated for its effect on capsule production in *V. vulnificus*. The appearance of colonies on LB agar plates of the wild type and mutant were similar (data not shown), indicating that *V. vulnificus* DM7 is able to produce

capsule as translucent colonies would be easily observed on LB agar plates. However, surprisingly, colonies of DM7 on DVNSS (low nutrient plates) were extremely mucoid when compared with the wild type strain, C7184(O) . These results indicate that the decrease in capsule production that occurs when cells are plated on low-nutrient media is due, either directly or indirectly to a functional SmcR.

***smcR* affects motility, fimbriation and biofilm formation**

During routine growth in LB broth, DM7 often appeared to clump. The present inventors therefore investigated the effect of the mutation on motility, fimbriae production and biofilm formation. In order to test the effect on motility, cells of C7184(O) and DM7 were inoculated into LB and cultured at 37°C with aeration to exponential phase. These cells were inoculated into motility agar (LB with 0.3% agar) (Bacto Difco agar, Difco Laboratories) as a stab in the center of plates and incubated 16 hours at 37°C. The rate of motility of the mutant strain was significantly faster than the wild type, with the mutant covering the plate within 16 hours . Similar results were obtained when motility agar plates were incubated at 30°C (data not shown).

In order to observe extracellular appendages on the wild type and mutant strain, electron microscopy was performed on stationary phase cells. Cells were grown overnight in LB at 37°C with aeration, washed in phosphate-buffered saline (PBS) and fixed by the addition of 2% glutaraldehyde to a final concentration of 1% (w/v). Nearly all of the wild type cells observed lacked fimbriae and flagella while the mutant cells usually possessed both . *V. vulnificus* has been reported to produce type IV pili (49) that are required for adherence to HEp-2 cells. The appearance of the surface appendages shown here is the same as that reported for the type IV pili.

Because fimbriae have been demonstrated to play a role in attachment and biofilm formation, the ability of the mutant to attach and form biofilms was tested. The *smcR* mutant strain formed more biofilm (507% of the wild type biofilm) in the microtiter plates than the wild type, which formed very little biofilm (Table 3). This increased biofilm formation by the mutant correlates with the electron microscopy data which demonstrates that the mutant has more fimbriae than the wild type.

**Exoenzyme production**

Various substrates were used to determine if the exoenzyme profile of the wild type is affected by the *smcR* null mutation. The present inventors observed no significant differences in hemolytic activity as assayed by overnight growth on blood agar as both the *V. vulnificus* C7184 and DM7 created similar zones of hemolysis (data not shown). Similarly, no significant differences were seen in siderophore production as assayed by the CAS assay (55) (data not shown).

Alkaline phosphatase activity was determined as described by the manufacturer (Sigma manual, Sigma Chemical Co., St. Louis MO). The alkaline phosphatase activity of supernatants obtained from cells grown in LB at 37°C for 24 hr is shown in Table 3. The results of a typical experiment are shown and are presented as alkaline phosphatase activity per cell ($OD_{540}$ nm/$OD_{610}$ nm) (in parentheses) and as a percentage of wild type activity. As can be seen, the *smcR* mutant produces 2.3 fold more alkaline phosphatase activity in an overnight culture than the wild type.

When the *smcR* mutant was assayed for exoprotease production, it was discovered that there is an earlier expression of protease activity and the final amount of protease in the supernatant during late stationary phase growth was always higher. Typical results for exoprotease expression of cells grown in LB at 37°C as determined by HPA, azocasein and elastin-Congo red substrate degradation are represented in Fig. 3. The results are presented as protease activity per cell and are plotted as a function of growth as determined by $OD_{610}$ nm readings. Similar trends in protease activities were obtained from supernatants collected from cultures grown at 24°C (data not shown). In all cases, the protease expression of the *smcR* mutant occurred earlier and the final activity was higher than for the wild type strain.

Because signals were implicated in the regulation of proteases, the present inventors tested the ability of the signal antagonist, furanone compound 2 (C2) to inhibit protease production (Table 3). Growth of the wild type strain in the presence of furanone compound 2 inhibited protease production, indicating that the signal transduction pathway is important for protease activity. Importantly, the concentration of C2 used was not growth inhibitory. In *V. cholerae*, a mutation in the *luxR* homologue, *hapR* resulted in a loss of HA/P expression (22). These results indicate that, unlike HapR in

*V. cholerae*, the gene product of *smcR* is involved in the repression of protease expression during exponential growth rather than for its induction.

**SmcR is involved in starvation survival and the SIMC response**

The phenotypes so far examined in the *smcR* mutant all are stationary-phase regulated. In order to determine whether the *smcR* gene product is involved in stationary phase survival, cells of *V. vulnificus* C7184(O) and DM7 were grown to early exponential phase in LB with 20 g $l^{-1}$ NaCl ($OD_{610}$ nm = 0.22), washed and resuspended in 2M-C. The results for samples maintained at room temperature are shown in Fig. 4A. There was an initial decrease of 76% in the CFUs for the mutant strain and no decrease for the wild type after 1 day. After 14 days of incubation at room temperature, the *smcR* mutant strain exhibited a decrease of 91% of the CFUs while the wild type only had a loss of 70% of CFUs. The loss in CFU for the two strains was not significantly different after the first 14 - 20 days of incubation. These results indicate that *smcR* is important for the maintenance of starvation survival, especially in the early stages (first 2 weeks) of starvation.

The defect in starvation survival exhibited by DM7 prompted an investigation of the effect of the *smcR* null mutation on the SIMC response. It has been previously demonstrated that starvation of *V. vulnificus* prior to low temperature incubation allows for the maintenance of culturability (SIMC) at low temperature (43, 48). This SIMC effect delays the formation of viable but nonculturable (VBNC) cells that typically occurs at temperatures below 10°C. This delay possibly allows cells to synthesize proteins that will be important in survival and recovery when conditions are again favorable. The results in Fig. 4B indicate that the *smcR* mutant strain is defective in mounting this protective maintenance of culturability response. The cultures that were shifted to 4°C without pre-starvation showed very little differences in the rate of loss of culturability. It should be noted that even the non-starved sample had been starved 30 min prior to shifting to 4°C due to the time required for washing, pelleting and resuspending the cells. The cultures that were starved at room temperature before cold incubation exhibited a difference in the rate of loss of culturability (Fig. 3B). By the third day of cold incubation, the mutant strain had lost 77% of the total CFUs while the wild type strain only showed a decrease of 28%. This trend continued throughout the cold incubation.

These data indicate that SmcR either directly or indirectly affects the pre-starvation response. To further investigate the hypothesis that the starvation response is signal-regulated, the present inventors tested the effect of the addition of the signal antagonist, C2, to inhibit the starvation response. Cells of C7184 were collected during early exponential phase, washed and resuspended in 2M with or without C2, and shifted to 4°C at time 0 and after 4 hr of starvation at room temperature. Results are shown in Fig. 4C. Starvation of cells in the presence of C2 at room temperature does not allow the delay in VBNC formation that occurs in the cultures in the absence of C2. These data further support the hypothesis that the signal system investigated here affects the starvation response.

**Discussion**

The majority of investigations of quorum-sensing systems endeavor to determine the mechanisms whereby bacteria are able to coordinate their phenotypic expression at the population level. In both the host - pathogen interaction, and in the external environment, sensing the quorum allows for coordination of gene expression at an appropriately high cell density. While this is the paradigm for some systems, such as the acylated homoserine lactone- (AHL) regulated signalling system, recent intriguing discoveries suggest that signal molecules may regulate phenotypes which are not density-dependent but are regulated in relation to growth phase or in response to local environmental conditions. For example, signal molecules have been shown to regulate the induction of stationary phase in *R. leuminosarum* (66) and *Pseudomonas aeruginosa* (75) and to induce the carbon starvation response in *V. angustum* (60). In addition, a quinolone signal discovered in *P. aeruginosa* has been shown to regulate one of the AHL systems in a density-independent manner during late stationary phase (30 - 42 hours post-inoculation) (36). These reports suggest there are density-independent signalling systems in some bacteria that regulate starvation and/or stationary phase phenotypes. The AI-2 system described here for *V. vulnificus* may be one such density-independent regulatory system and furthermore this system may be an important global regulator of adaptation to physiological changes or stresses.

*V. vulnificus* produces an extracellular, polar molecule that induces luminescence in *V. harveyi* BB170, which has the signal- sensing phenotype

sensor $1^-$, sensor $2^+$ and is therefore only induced via the system 2 signalling pathway. Two strains of *V. vulnificus* were demonstrated to produce this signal at 37°C and 24°C as well as in complex and minimal media, while the addition of glucose to complex media results in inhibition of signal production. Maximal signal production occurs upon entry into the stationary phase of growth. The most interesting feature of signal production in *V. vulnificus* is its induction in cells, which have been shifted from exponential growth to starvation conditions.

Furthermore, the present inventors have identified genes in *V. vulnificus*, *smcR* and *luxS*, that are apparent homologues of signal-related genes in *V. harveyi* (34). All six of the *Vibrio* strains tested, *Vibrio anguillarum, V. angustum, V. alginolyticus, V. vulnificus, V. cholerae,* and *V. harveyi*, contained homologues of the *smcR* gene. The amino- and carboxy-terminal translated regions of the four reported *luxR* homologues are similar for *V. harveyi, V. vulnificus, V. cholerae*, and *V. parahaemolyticus*. All four of the *luxR* ORFs described have a divergently transcribed homologue of *hpt* (hypoxanthine ribosyltransferase) located upstream. In *V. vulnificus* and *V. parahaemolyticus*, the ORFs are followed by a homologue of *lpd* (dihydrolipoamide dehydrogenase). Coupled with the identification of an extra-cellular, polar factor that stimulates the *V. harveyi* AI-2 responder, it would appear that *V. vulnificus* has an AI-2 signalling pathway. The identification of AI-2 signals and signal related genes in this organism and many others would suggest that the AI-2 pathway is widely dispersed amongst bacteria. For example, the induction of luminescence in *V. harveyi* BB170 has been reported for *Yersinia* (2) and highly conserved *luxS* homologues have been discovered in a wide range of Gram-negative and Gram-positive bacteria (62) including *Mycobacterium, Bacillus, Pasteurella* and *Borrelia*. Similarly, phylogenetic analysis of these genes identified in organisms as widely dispersed as *V. harveyi* and *Bacillus* (34, 62), would suggest that the genes needed to accomplish the AI-2-mediated signalling pathway are an ancient system and may have been present since these organisms diverged.

While the general features of the AI-2 systems appear to be highly conserved across a broad range of genera and species, some of the specific features of the system clearly differ and may reflect individual adaptation of the AI-2 system to the specific needs of particular bacteria. In *E. coli* for

example, it has been reported that at least one extracellular signal is resistant to pH 12 for 20 min, 100°C for 10 min and is produced in LB without glucose (71) during the entry into stationary phase. However, it has also been reported that an extracellular signal molecule produced by some strains of *E. coli* and *S. typhimurium* and able to induce the *V. harveyi* AI-2 monitor strain is only produced in LB with the addition of glucose in exponential phase and is inactivated at 100°C (61). The factor produced by *V. vulnificus* is heat labile at 100°C but is produced during late exponential and early stationary phase. Perhaps the most interesting feature of the signal produced by *V. vulnificus* is its repression by glucose addition, which clearly suggests that there are differences in the regulation of production of these molecules amongst different bacteria. These data indicate that there may be more than one AI-2 factor, where the AI-2 factors produced by different bacteria or strains of bacteria share a basic structure but also are slightly different. For example, there are a range of AHL molecules that vary by the length of the acyl chain (e.g. C6-HSL, and C4-HSL), substitution at the third carbon on the chain (e.g. C6-HSL and 3-oxo C6 HSL), or by the degree of saturation of the acyl chain (e.g. 3-OH, C14:1 HSL) and these modifications of the basic AHL structure may contribute to vastly different physical or chemical properties of the signal molecules.

A mutation in the *smcR* gene does not affect the ability of the cells to produce signal molecules. This would suggest that the production of the AI-2 signal is not regulated via an auto-induction circuit in *V. vulnificus*. Therefore, signal production may be controlled through other regulators, such as those that regulate entry into stationary phase or signal production may be constitutive and the response to the signal may be controlled in a growth phase dependent fashion.

In *V. parahaemolyticus*, disruption of *opaR*, a homologue of the *V. harveyi luxR*, in an opaque genetic background results in transformation of the strain to the translucent morphotype (32). *V. vulnificus* also experiences the opaque to translucent conversion; in addition, the expression of capsular polysaccharide (CPS) is environmentally regulated (73). Cell surface expression of CPS in wild types strain of *V. vulnificus* varies with growth phase, increasing during logarithmic growth and declining in stationary phase (73). The timing of CPS repression in those studies (73) correlates with the time where we observe increases in signal production. Unlike *V.*

*parahaemolyticus*, however, a null mutation in *smcR* results in an increase in capsule production under some growth conditions. It is therefore possible that *smcR* is responsible, either directly or indirectly, for the decrease in CPS production seen in stationary phase cells. In El Tor strains of *V. cholerae*, deletion of the *luxR* homologue, *hapR*, leads to conversion of the translucent morphotype to the opaque or rugose morphotype (22). This data would suggest that *hapR* acts as a repressor of CPS production, which appears to be similar to the effects observed for CPS production as well as other phenotypes in *V. vulnificus*.

Indeed, it has been previously suggested that LuxR may function as a repressor. For example, LuxR binds independently to two sites upstream of its own open reading frame (6). Results from experiments using a *cat* transcriptional fusion to the *luxR* promoter reveal a repression of transcription from the *luxR* promoter as a result of possibly interfering with and displacing RNA polymerase from the promoter (6). Furthermore, LuxR has not been shown to have an activator domain. Transcriptional run-off experiments have shown that *luxR* expression is required to alter the transcription start site of the *lux* operon from the -123 location to the -26 location by repressing transcription from -123 but has not been shown to induce transcription from the -26 site (63). This would indicate that LuxR induces the *lux* operon by repressing one of the transcription start sites, thereby shifting RNA polymerase binding to the -26 site. LuxR is a member of the TetR family of transcriptional regulators which act as repressors (18). Taken together, these data indicate that the primary function of the LuxR regulator, at least in some organisms, may in fact be the repression of gene transcription rather than activation. It is clear, however, that in *V. cholerae* and *V. parahaemolyticus*, LuxR acts as an activator of protease expression and capsule production respectively. SmcR in *V. vulnificus* appears to act both as a repressor and as an activator by repressing vegetative gene expression in exponential phase, while activating stationary phase gene expression. Thus, SmcR may function in a fashion similar to the *E. coli* regulatory proteins TyrR (51) or to nitrogen regulator I ($NR_I$) (53), both of which have been demonstrated to both activate and repress genes in their regulons.

The possibility that LuxR can function as a repressor fits with some of the phenotypes observed in the *smcR* mutant in *V. vulnificus*. For example, deletion of the gene leads to an up-regulation of protease production, alkaline

phosphatase production, fimbriation, motility and biofilm formation. In the wild-type strain, all of these phenotypes are induced at the onset of or early in stationary phase. In support of the hypothesis that SmcR acts as a repressor, the mapping of the time of induction of enzyme activity also occurs earlier than in the wild type, suggesting that the mutant is impaired in the timing of stationary-phase gene expression. If the *smcR* is acting as a repressor, it may function to repress stationary phase phenotypes in exponential phase, and this repression may be relieved at the onset of stationary phase or upon nutrient deprivation. It seems likely that signal production and recognition, which occurs at the transition into stationary phase or shortly after entry into starvation, when these phenotypes are normally expressed, may be the mediator of this relief of repression.

Not only is *smcR* important for the regulation of protease and alkaline phosphatase, but the data presented here reveals that it plays an important role in starvation adaptation and the viable but nonculturable (VBNC) response, possibly through gene activation. *V. vulnificus* enters a nonculturable state when exposed to low temperature (35, 43, 44). Entrance into the VBNC state is related to the physiological state of the cells as pre-starvation delays entrance into the VBNC state (43, 48). This starvation-induced maintenance of culturability (SIMC) is dependent on the production of carbon starvation-induced proteins. It has been demonstrated in numerous species that starvation-induced protein synthesis prepares cells for survival during stress and may be important in the reactivation of cells when conditions are again favorable (21, 31). In the natural environment, it is likely that a proportion of *V. vulnificus* cells would encounter nutrient limitation prior to low temperature conditions. The production of these starvation proteins may act to delay VBNC formation until the cells have become more stress-resistant, thereby providing the cells with a greater chance of survival during low temperature incubation and by increasing the chance of resuscitation upon an increase in temperature. The loss of a functional *smcR* prevents *V. vulnificus* from exhibiting a full SIMC response upon starvation prior to low temperature incubation. In addition, the *smcR* mutant was defective in starvation survival at room temperature, exhibiting a 76% loss of CFUs after 24 incubation in comparison to the wild type, which showed 100% culturability. This is the first report of the regulation of starvation adaptation by a *V. harveyi luxR* homologue. In addition to the

defects in starvation adaptation that were discovered, as described above, the *smcR* mutant is also derepressed for numerous genes that are normally induced during the stationary phase of growth. Therefore, it would appear that, based on analogies with other global regulators, SmcR is also a mediator of stationary phase physiology and starvation adaptation as well as a regulator of virulence factor production.

Furanone compounds produced by the red marine alga *Delisea pulchra*, have the ability to act as signal antagonists, thereby inhibiting signal-dependent phenotypes (11, 12, 30, 60). The present inventors have shown that the addition of furanone compound 2 does not inhibit the production of signal molecules in *V. vulnificus*. Furanone compound 2 does, however, inhibit the expression of a major virulence factor in *V. vulnificus*, the signal-regulated metalloprotease and blocks the starvation-mediated maintenance of culturability at low temperature. The observation that a signal antagonist has the ability to inhibit phenotypes such as protease expression and the development of SIMC further supports the suggestion that these are signal-related phenotypes.

Due to the pleiotrophic effects of the *smcR* mutation in *V. vulnificus*, it is tempting to speculate that SmcR acts either as a global regulator of stationary phase genes or that SmcR controls other global regulators, such as the stationary phase sigma factor, RpoS. It is also possible that SmcR acts through other pathways, such as the ToxR signal transduction system. It has been shown that ToxR regulates expression of type IV fimbriae in *V. cholerae* and that ToxR mutants exhibit increases in swarming, protease production and attachment to HEp-2 cells (10). Whatever the mechanism of control, it is apparent that the signalling system 2 investigated here is involved, either directly or indirectly, in the regulation of density-independent gene expression and regulates starvation and stationary phase phenotypes. This discovery expands the role of signalling systems to include global regulation of non-growth physiology.

TABLE 1. Bacterial strains and plasmids

| Strain or Plasmid | Description | Reference or source |
|---|---|---|
| Strains | | |
| *V. vulnificus* | | |
| C7184(O) | Human wound isolate; encapsulated and virulent | (46) |
| C7184(T) | Non-encapsulated spontaneous mutant of C7814(O) | (58) |
| DM7 | *smcR*::Sm, derived from C7184(O) | Described herein |
| UTHS-1 | | (72) |
| *V. harveyi* | | |
| BB170 | *luxN*::Tn*5* | (3) |
| BB152 | *luxL*::Tn*5* | (3) |
| *E. coli* | | |
| BW20767 | $Sm^r$, (RP4-2 *tet* : Mu-1*kan*::Tn*7* integrant) *tra+ leu-63*::IS10 *recA1 creC510 hsdR17 endA1 zbf-5 uidA(Mlu1):pir+ thi* | (37) |
| DH5α | *supE44 ΔlacU169* (ϕ80*lacZ*ΔM15)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1* | (16) |
| XLI Blue | *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F proAB lacI$^q$ZDM15* Tn*10(tet$^r$)*] | (4) |
| *Plasmids* | | |
| pBluescript® II SK+ | $Ap^r$, multiple cloning site flanked by T3 and T7 promoters, $p_{lac}$ promoter fused to the α peptide of 'LacZ, derived from pUC19 | Stratagene |
| pCAM140 | $Sm^r$, $Ap^r$, mini-Tn*5* *gusA* in pUT mini T*n5* Sm/Sp | (70) |
| pCVD442 | $Ap^r$, positive selection vector, pGP704 with *sacB* inserted in multiple cloning site | (8) |
| pLG401 | $Cm^r$, pACYC ori, mob, promoterless *gfp* with multiple cloning site | Lynn Gilson, University of Hawaii |
| pMacSB | $Cm^r$, *sacB* from pCVD442 inserted as *Pst*I-*Eco*RV fragment into cloning site of | Described herein |

| Strain or Plasmid | Description | Reference or source |
|---|---|---|
| | pLG401 | |
| pMacSmcRK | pMacSB with disrupted *smcR* fragment from SprA.SM inserted into *Eco*RV site of pMacSB | Described herein |
| pUC19 | $Ap^r$, multiple cloning site, $p_{lac}$ promoter fused to the α peptide of 'LacZ | (74) |
| pSmcR8 | $Ap^r$, pUC19 with 720 bp insert containing the *V. vulnificus smcR* | Described herein |
| pSmcR8.18 | $Ap^r$, pBluescript with ~770 bp insert containing the *Eco*R1-*Hind*III *smcR* fragment from pSprA8 | Described herein |
| pSmcR.SM | $Ap^r$, $Sm^r$, pSprA8.18 with ~2.0 kb Sm fragment from pCAM140 inserted into the *Bgl*II site of the *smcR* insert | Described herein |

TABLE 2. Induction of luminescence in *V. harveyi* reporter strain by cell-free supernatants collected from *V. vulnificus*

| Species and conditions | Induction of luminescence (%) in *V. harveyi* reporter strain |
|---|---|
| *V. harveyi* BB152[a] | |
| AB | 100.0 |
| *V. vulnificus* C7184(T)[b] | |
| 2M | 136.8 |
| 80°C treated[c] | 72.1 |
| 100°C treated[c] | 4.8 |
| Grown with furanone compound 2 | 152.0 |
| LB | 146.4 |
| LB + glucose | 5.7 |
| *E. coli* DH5α | |
| LB + glucose | 3.45 |

[a]Luminescence induced by *V. harveyi* BB152 supernatant was normalized to 100%

[b]Grown at 37°C in medium indicated and collected at entry into stationary phase

[c]Cell-free supernatants were incubated at temperature indicated for 10 min

TABLE 3. Selected phenotypes of a *V. vulnificus smcR* mutant

| Strain of *V. vulnificus* | Signal production[a] | Alkaline phosphatase[b] | Biofilm formation[c] | Protease activity[d] |
|---|---|---|---|---|
| DM7 (*smcR*) | + | 230 (1.2) | 507.4 | 121.4 (0.227) |
| C7184(O) (*smcR*$^+$) | + | 100 (0.521) | 100.0 | 100 (0.187) |
| C7184(O) + C2[e] | + | ND[f] | ND | $1x10^{-3}$ (0.032) |

[a]Cell-free supernatants were collected from early stationary phase cultures and their ability to induce luminescence in the *V. harveyi* reporter strain tested

[b]Alkaline phosphatase production as a percentage of the wild type activity and as activity per cell (parentheses) is shown

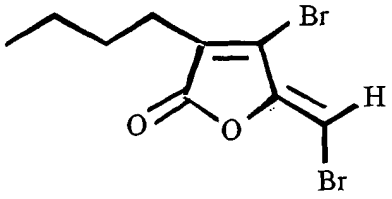
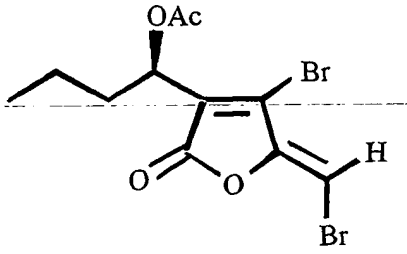
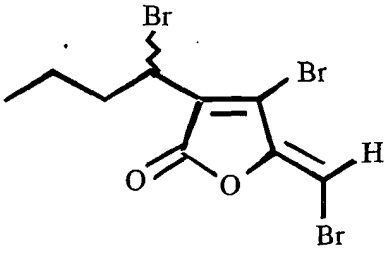
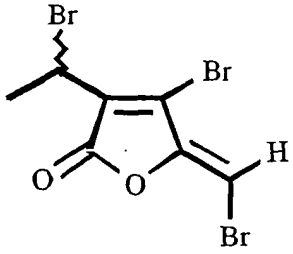
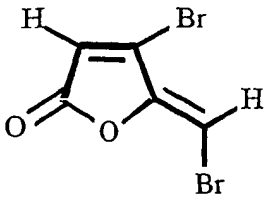
[c]Biofilm formation at 24 hr in microtiter plates presented as percent of wild type attachment

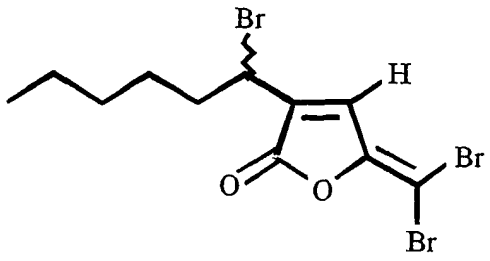
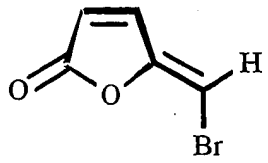
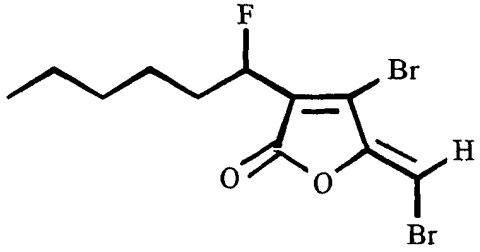
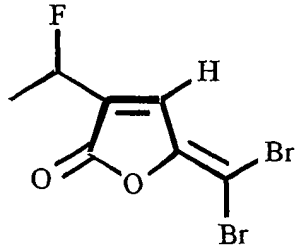
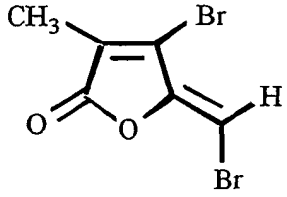
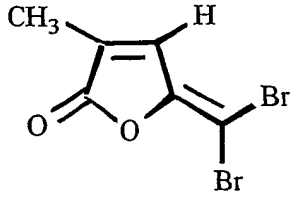
[d]Protease production as a percentage of the wild type HPA activity and as activity per cell (parentheses) is shown

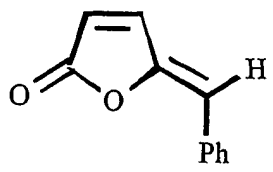
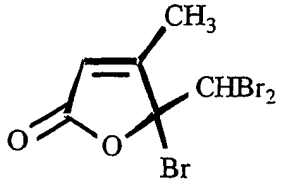
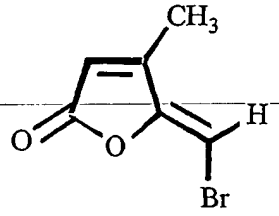
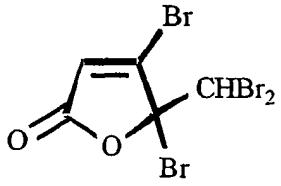
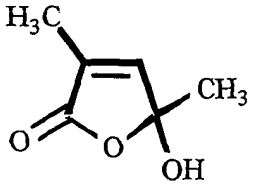
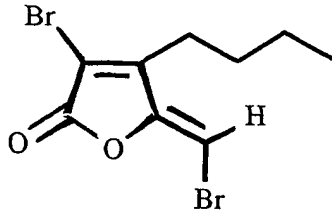
[e]Furanone compound 2 was added at a concentration of 10 $\mu$g ml$^{-1}$ during mid exponential phase and the culture incubated at 37°C for 4 hr (stationary phase) prior to collection of supernatants

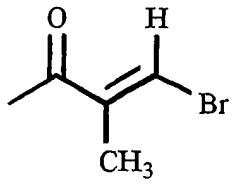
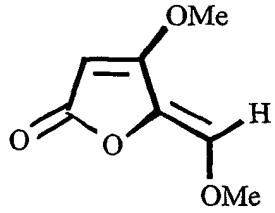
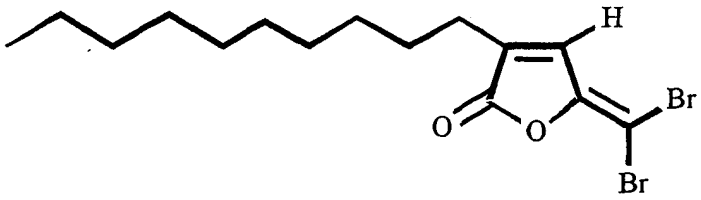
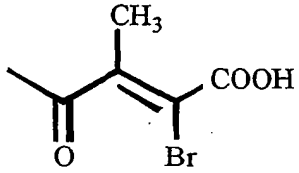
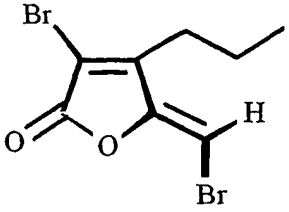
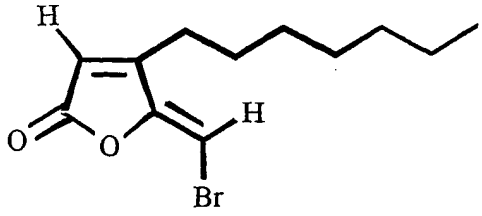
[f]ND, not determined

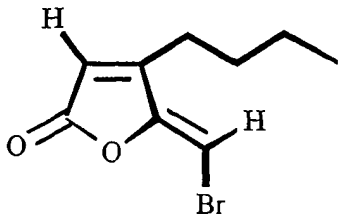
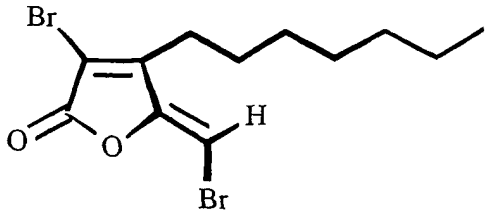
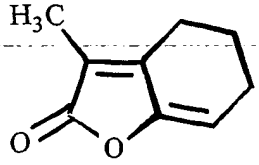
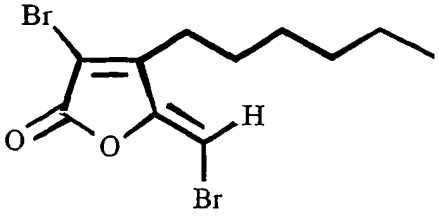
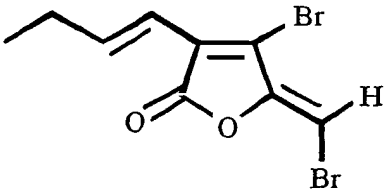
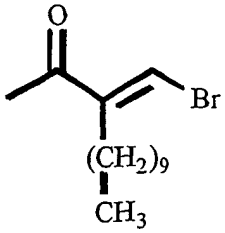
Table 4

| Compound | Structure |
|---|---|
| **2 (d3)** |  |
| **3 (d5)** |  |
| **24** |  |
| **26** |  |
| **30** |  |

| | |
|---|---|
| 34 |  |
| 56 |  |
| 57 |  |
| 60 |  |
| 63 |  |
| 64 |  |

| | |
|---|---|
| 70 |  |
| 72 |  |
| 73 |  |
| 74 |  |
| 75 |  |
| 78 |  |

| | |
|---|---|
| 80 |  |
| 85 |  |
| 88 |  |
| 91 |  |
| 99 |  |
| 102 |  |

| | |
|---|---|
| 103 |  |
| 105 |  |
| 112 |  |
| 113 |  |
| 118 |  |
| 124 |  |

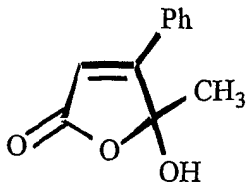
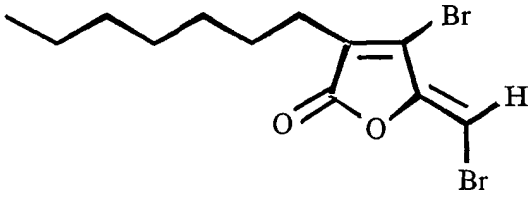
| | |
|---|---|
| 129 |  |
| 139 |  |

Table 5. The effects of furanones against the AI-2 specific V. harveyi bioluminescence monitor

| Compound | Concentration (ug/ml) | % Luminescence of the control | Growth inhibition |
|---|---|---|---|
| 2 | 1 | 168 | No |
| | 25 | 0.4 | Yes |
| 3 | 50 | 69 | Not tested |
| | 500 | ND* | Not tested |
| 19 | 25 ug/ml | 50% | No |
| 24 | 5 | 3 | Not tested |
| | 25 | 0.6 | Not tested |
| | 50 | 0.1 | Not tested |
| 26 | 5 | 5 | Not tested |
| | 25 | 1.1 | Not tested |
| | 50 | 0.3 | Not tested |
| 30 | 1 | 0.8 | No |
| | 10 | 0.6 | Yes |
| | 25 | 0.1 | Yes |
| 34 | 1 | 321 | No |
| | 10 | 0.2 | Yes |
| | 25 | 0.5 | Yes |
| 70 | 1 | 0.178 | No |
| | 10 | 0.725 | No |
| | 25 | 0.221 | Yes |
| 72 | 1 | 8.1 | No |
| | 10 | 0.447 | No |
| | 25 | 0.366 | Yes |
| 74 | 1 | 170.2 | No |
| | 10 | 0.377 | Yes |
| | 25 | 0.25 | Yes |
| 75 | 1 | 94 | No |
| | 10 | 123 | No |
| | 25 | 101 | No |
| 76 | 1 | 110 | No |

| Compound | Concentration (ug/ml) | % Luminescence of the control | Growth inhibition |
|---|---|---|---|
| | 10 | 90 | No |
| | 25 | 87 | No |
| 77 | 25 ug/ml | >99% | No |
| 78 | 1 | 2.5 | Slight |
| | 10 | ND | Yes |
| | 25 | ND | Yes |
| 80 | 10 ug/ml, | 25%, | No |
| | 1 ug/ml | 50% | No |
| 85 | 1 | 65 | No |
| | 10 | 74 | No |
| | 25 | 92 | No |
| 88 | 1 | 110 | No |
| | 10 | 100 | No |
| | 25 | 85 | No |
| 91 | 1 | 15 | No |
| | 10 | 35 | No |
| | 25 | 107 | No |

*ND, below detection limit

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

References

1. **Bassler, B. L.** 1999. How bacteria talk to each other: regulation of gene expression by quorum sensing. Curr. Opin. Microbiol. **2**:582-587.

2. **Bassler, B. L., E. P. Greenberg, and A. M. Stevens.** 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J. Bacteriol. **179**:4043-4045.

3. **Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman.** 1993. Intercellular signalling in *Vibrio harveyi* : sequence and function of genes regulating expression of luminescence. Mol. Microbiol. **9**:773-786.

4. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. X 1-blue: A high efficiency plasmid transforming *recA Escherichia coli* strain with Beta-galactosidase selection. Biotechniques **5**:376-379.

5. **Chang, T. M., Y. C. Chuang, J. H. Su, and M. C. Chang.** 1997. Cloning and sequence analysis of a novel hemolysin gene (*vllY*) from *Vibrio vulnificus*. Appl. Environ. Microbiol. **63**:3851-3857.

6. **Chatterjee, J., C. M. Miyamoto, and E. A. Meighen.** 1996. Autoregulation of *luxR*: the *Vibrio harveyi lux*-operon activator functions as a repressor. Mol. Microbiol. **20**:415-425.

7. **Church, G. W., and W. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci., USA **81**:1991-1995.

8. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59**:4310-4317.

9. **Gander, R. M., and M. T. Larocco.** 1989. Detection of pilus-like structures on clinical and environmental isolates of *Vibrio vulnificus*. J. Clin. Microbiol. **27**:1015.

10. **Gardel, C. L., and J. J. Mekalanos.** 1996. Alterations in *Vibrio cholerae* motility phenotypes correlate with changes in virulence factor expression. Infect. Immun. **64**:2246-2255.

11. **Givskov, M., R. DeNys, M. Manefield, L. Gram, R. Maximilien, L. Eberl, S. Molin, P. D. Steinberg, and S. Kjelleberg.** 1996. Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. J. Bacteriol. **178**:6618-6622.

12. **Gram, L., R. DeNys, R. Maximilien, M. Givskov, P. Steinberg, and S. Kjelleberg.** 1996. Inhibitory effects of secondary metabolites from the red alga

*Delisea pulchra* on swarming motility of *Proteus mirabilis*. Appl. Environ. Microbiol. **62**:4284-4287.

13. **Gray, K. M., J. P. Pearson, J. A. Downie, B. E. A. Boboye, and E. P. Greenberg.** 1996. Cell-to-cell signalling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum* : autoinduction of a stationary phase and rhizosphere-expressed genes. J. Bacteriol. **178**:372-376.

14. **Gray, L. D., and A. S. Kreger.** 1985. Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. Infect. Immun. **48**:62-72.

15. **Greenberg, E. P., J. W. Hastings, and S. Ulitzer.** 1979. Induction of luciferase synthesis in *Beneckea harveyi* by other marine bacteria. Arch. Microbiol. **120**:87-91.

16. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557-580.

17. **Hanne, L. F., and R. A. Finkelstein.** 1982. Characterization and distribution of the hemagglutinins produced by *Vibrio cholerae*. Infect. Immun. **36**:209-214.

18. **Hinrichs, W., C. Kiser, M. Düvel, A. Müller, K. Tovar, W. Hillen, and W. Saenger.** 1994. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. Science **264**:418-420.

19. **Hoben, H. J., and P. Somasegaran.** 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl. Environ. Microbiol. **44**:1246-1247.

20. **Høi, L., J. L. Larsen, I. Dalsgaard, and A. Dalsgaard.** 1998. Occurrence of *Vibrio vulnificus* biotypes in Danish marine environements. Appl. Environ. Microbiol. **64**:7-13.

21. **Jenkins, D. E., J. E. Schultz, and A. Matin.** 1988. Starvation-induced cross protection against heat or H2O2 challange in *Escherichia coli*. J. Bacteriol. **170**:3910-3914.

22. **Jobling, M. G., and R. K. Holmes.** 1997. Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene *hap*, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. Mol. Microbiol. **26**:1023-1034.

23. **Kaspar, C. W., and M. L. Tamplin.** 1993. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl. Environ. Microbiol. **59**:2425-2429.

24. **Kothary, M. H., and A. S. Kreger.** 1985. Production and partial characterization of an elastolytic protease of *Vibrio vulnificus*. Infect. Immun. **50**:534-540.

25. **Kothary, M. H., and A. S. Kreger.** 1987. Purification and characterization of an elastolytic protease of *Vibrio vulnificus*. J. Gen. Microbiol. **133**:1783-1791.

26. **Kreger, A., L. DeChatelet, and P. Shirley.** 1981. Interaction of *Vibrio vulnificus* with human polymorphonuclear leukocytes: association of virulence with resistance to phagocytosis. J. Infect. Dis. **144**:244-248.

27. **Kreger, A., and D. Lockwood.** 1981. Detection of extracellular toxin(s) produced by *Vibrio vulnificus*. Infect. Immun. **33**:583-590.

28. **Litwin, C. M., T. W. Rayback, and J. Skinner.** 1996. Role of catechol siderophore synthesis in *Vibrio vulnificus* virulence. Infect. Immun. **64**:2834-2838.

29. **Manefield, M., R. DeNys, N. Kumar, R. Read, M. Givskov, P. Steinberg, and S. Kjelleberg.** 1999. Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology **145**:283-291.

30. **Manefield, M., L. Harris, S. A. Rice, R. DeNys, and S. Kjelleberg.** 2000. Halogenated furanones from *Delisea pulchra* inhibit bioluminescence and virulence in the *Paneus monodon* pathogen *Vibrio harveyi*. Appl. Environ. Microbiol. **66**:2079-2084.

31. **Matin, A.** 1991. The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. Mol. Microbiol. **5**:3-10.

32. **McCarter, L. L.** 1998. OpaR, a homolog of *Vibrio harveyi* LuxR, controls opacity of *Vibrio parahaemolyticus*. J. Bacteriol. **180**:3166-3173.

33. **McDougald, D., S. A. Rice, and S. Kjelleberg.** 1999. New perspectives on the viable but nonculturable response. Biologia **54**:617-623.

34. **McDougald, D., S. A. Rice, and S. Kjelleberg.** 2000. *Vibrio vulnificus* encodes a putative homologue of the *Vibrio harveyi* regulatory gene, *lux*R: a genetic and phylogenetic comparison. Gene **248**:213-221.

35. **McDougald, D., S. A. Rice, D. Weichart, and S. Kjelleberg.** 1998. Nonculturability: adaptation or debilitation? FEMS Microbiol. Ecol. **25**:1-9.

36. **McKnight, S. L., B. H. Iglewski, and E. C. Pesci.** 2000. The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*. J. Bacteriol. **182**:2702-2708.

37. **Metcalf, W. W., W. Jiang, L. L. Daniels, S.-K. Kim, A. Haldimann, and B. L. Wanner.** 1996. Conditionally replicative and conjugative plasmids carrying *lacZα* for cloning, mutagenesis, and allele replacement in bacteria. Plasmid **35**:1-13.

38. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Springs Harbor Laboratory Press, Cold Spring Harbor, NY.

39. **Miyoshi, S., H. Nakazawa, K. Kawata, K. Tomochika, K. Tobe, and S. Shinoda.** 1998. Characterization of the hemorrhagic reaction caused by *Vibrio vulnificus* metalloprotease, a member of the thermolysin family. Infect. Immun. **66**:4851-4855.

40. **Miyoshi, S.-i., Y. Hirata, K.-i. Tomochika, and S. Shinoda.** 1994. *Vibrio vulnificus* may produce a metalloprotease causing an edematous skin lesion in vivo. FEMS Microbiol. Lett. **121**:321-326.

41. **Novick, R. P.** 1999. Regulation of pathogenicity in *Staphylococcus aureus* by a peptide-based density-sensing system, p. 129-146. *In* G. M. D. a. S. C. Winans (ed.), Cell-Cell Signalling in Bacteria. American Society for Microbiology, Washington, D. C.

42. **Okujo, N., T. Akiyama, S. Miyoshi, S. Shinoda, and S. Yamamoto.** 1996. Involvement of vulnibactin and exocellular protease in utilization of transferrin- and lactoferrin-bound iron by *Vibrio vulnficus*. Microbiol. Immunol. **40**:595-.

43. **Oliver, J. D.** 1991. Formation of nonculturable *Vibrio vulnificus* cells and its relationship to the starvation state. Appl. Environ. Microbiol. **57**:2640-2644.

44. **Oliver, J. D.** 1993. Formation of viable but nonculturable cells, p. 239-272. *In* S. Kjelleberg (ed.), Starvation in Bacteria. Plenum Press, New York.

45. **Oliver, J. D.** 1995. The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol. Lett. **133**:203-208.

46. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1982. Distribution and ecology of *Vibrio vulnificus* and other lactose-fermenting marine vibrios in coastal waters of the southeastern United States. Appl. Environ. Microbiol. **44**:1404-1414.

47. **Oliver, J. D., J. E. Wear, M. B. Thomas, M. Warner, and K. Linder.** 1986. Production of extracellular enzymes and cytotoxicity by *Vibrio vulnificus*. Diagn. Microbiol. Infect. Dis. **5**:99-111.

48. **Paludan-Müller, C., D. Weichart, D. McDougald, and S. Kjelleberg.** 1996. Analysis of starvation conditions that allow for prolonged culturability of *Vibrio vulnificus* at low temperature. Microbiology **142**:1675-1684.

49. **Paranjpye, R. N., J. C. Lara, J. C. Pepe, C. M. Pepe, and M. S. Strom.** 1998. The type IV leader peptidase/*N*-methyltransferase of *Vibrio vulnificus* controls factors required for adherence to HEp-2 cells and virulence in iron-overloaded mice. Infect. Immun. **66**:5659-5668.

50. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260**:1127-1130.

51. **Pittard, A. J., and B. E. Davidson.** 1991. TyrR protein of *Escherichia coli* and its role as repressor and activator. Mol. Microbiol. **5**:1585-1592.

52. **Poole, M. D., J. H. Bowdre, and D. Klapper.** 1982. Presented at the Annual Meeting of the American Society for Microbiology.

53. **Reitzer, L. J., and B. Magasanik.** 1985. Expression of *glnA* in *Escherichia coli* is regulated at tandem promoters. Proc. Natl. Acad. Sci., USA **82**:1979-1983.

54. **Rodrigues, D. P., R. V. Ribeiro, and E. Hofer.** 1992. Analysis of some virulence factors of *Vibrio vulnificus* isolated from Rio de Janeiro, Brazil. Epidemiol. Infect. **108**:463-467.

55. **Schwyn, B., and J. B. Neilands.** 1986. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160**:47-56.

56. **Shin, M. G., J. H. Shin, S. P. Suh, D. W. Ryang, and K. S. Bae.** 1997. Cellular fatty acid profiles of ninety-five strains of *Vibrio vulnificus* isolated from clinical specimens in Korea. J. Gen. Appl. Microbiol. **43**:317-324.

57. **Simpson, L. M., and J. D. Oliver.** 1983. Siderophore production by *Vibrio vulnificus*. Infect. Immun. **41**:644-649.

58. **Simpson, L. M., V. K. White, S. F. Zane, and J. D. Oliver.** 1987. Correlation between virulence and colony morphology in *Vibrio vulnificus*. Infect. Immun. **55**:269-272.

59. **Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper.** 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and

enteropathogenic *Escherichia coli*. Proc. Natl. Acad. Sci., USA **96:**15196-15201.

60. **Srinivasan, S., J. Ostling, T. Charlton, R. de Nys, K. Takayama, and S. Kjelleberg.** 1998. Extracellular signal molecule(s) involved in the carbon starvation response of marine *Vibrio* sp. strain S14. J. Bacteriol. **180:**201-209.

61. **Surette, M. G., and B. L. Bassler.** 1998. Quorum sensing in *Escherichia coli* and *Salmonella typhimurium*. Proc. Natl. Acad. Sci., USA **95:**7046-7050.

62. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli, Salmonella typhimurium,* and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci., USA **96:**1639-1644.

63. **Swartzman, E., and E. A. Meighen.** 1993. Purification and characterization of a poly(dA-dT) *lux*-specific DNA-binding protein from *Vibrio harveyi* and identification as LuxR. J. Biol. Chem. **268:**16706-16716.

64. **Testa, J., L. W. Daniel, and A. S. Kreger.** 1984. Extracellular phospholipase A2 and lysophospholipase produced by *Vibrio vulnificus*. Infect. Immun. **45:**458-463.

65. **Thampuran, N., and P. K. Surendran.** 1998. Occurrence and distribution of *Vibrio vulnificus* in tropical fish and shellfish from Cochin (India). Lett. Appl. Microbiol. **26:**110-112.

66. **Thorne, S. H., and H. D. Williams.** 1999. Cell density-dependent starvation survival of *Rhizobium leguminosarum* bv. phaseoli: identification of the role of an *N*-acyl homoserine lactone in adaptation to stationary-phase survival. J. Bacteriol. **181:**981-990.

67. **Tillett, D., and B. Neilan.** 2000. Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. J. Phycol. **36:**1-8.

68. **Veenstra, J., P. J. G. M. Rietra, J. M. Coster, E. Slaats, and S. Dirks-Go.** 1994. Seasonal variations in the occurrence of *Vibrio vulnificus* along the Dutch coast. Epidemiol. Infect. **112:**285-290.

69. **von Bodman, S. B., and S. K. Farrand.** 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. J. Bacteriol. **177:**5000-5008.

70. **Wilson, K. J., A. Sessitsch, J. C. Corbo, K. E. Giller, A. D. L. Akkermans, and R. A. Jefferson.** 1995. B-glucuronidase (GUS) transposons for ecological and genetic studies of rhizobia and other Gram-negative bacteria. Microbiology **141:**1691-1705.

71. **Withers, H. L., and K. Nordström.** 1998. Quorum-sensing acts at initiation of chromosomal replication in *Escherichia coli*. Proc. Natl. Acad. Sci., USA **95**:15694-15699.

72. **Wolf, P. W., and J. D. Oliver.** 1992. Temperature effects on the viable but nonculturable state of *Vibrio vulnificus*. FEMS Microbiol. Ecol. **101**:33-39.

73. **Wright, A. C., J. L. Powell, M. K. Tanner, L. A. Ensor, A. B. Karpas, J. G. J. Morris, and M. B. Sztein.** 1999. Differential expression of *Vibrio vulnificus* capsular polysaccharide. Infect. Immun. **67**:2250-2257.

74. **Yanisch-Perron, C., J. Vieria, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103-119.

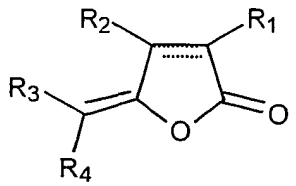
75. **You, Z., J. Fukushima, K. Tanaka, S. Kawamoto, and K. Okuda.** 1998. Induction of entry into stationary growth phase in *Pseudomonas aeruginosa* by *N*-acylhomoserine lactone. FEMS Microbiol. Lett. **164**:99-106.

CLAIMS:

1. A method of inhibiting a virulence factor in a microorganism comprising exposing the microorganism to a substance that inhibits a pathway that regulates the expression of the virulence factor.

2. A method according to claim 1, wherein the pathway is an AI-2 signalling system pathway.

3. A method of inhibiting a virulence factor in a microorganism, the method comprising exposing the microorganism to a composition comprising an effective concentration of a compound of general formula II:



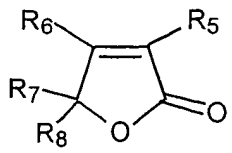
II

wherein $R_1$ and $R_2$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_3$ and $R_4$ are independently H, halogen, alkyl, aryl or arylalkyl, alkoxy, alkylsilyl or $R_3$ or $R_4$ + $R_2$ is a saturated or an unsaturated cycloalkane; and

"-------" represents a single bond or a double bond,

or a compound of general formula III:



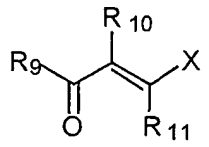
III

wherein $R_5$ and $R_6$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted,

optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_7$ and $R_8$ are independently H, halogen, hydroxy, alkyl, haloalkyl, dihaloalkyl, trihaloalkyl, aryl, arylalkyl, a hydroxy compound of a derivative of a hydroxy compound; or $R_7$ or $R_8$ + $R_6$ is saturated or unsaturated cycloalkane,

or a compound of general formula IV




IV

wherein $R_{10}$ and $R_{11}$ are independently H, halogen, carboxyl, ester, formyl, cyano, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

X is a halogen; and

$R_9$ is H, alkyl, alkenyl, alkynyl, alkene, alkyne, aryl, arylalkyl, whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic.

4. A method according to any one of the preceding claims, wherein the virulence factor is a microbial product or a physiological characteristic that causes disease or pathology in an animal.

5. A method according to any one of the preceding claims, wherein the virulence factor is an AI-2 mediated phenotype.

6. A method according to any one of the preceding claims, wherein the virulence factor is selected from the group consisting of exoprotease production, capsule production, biofilm formation, surface colonisation, toxin production and/or secretion, attachment, surface translocation, stress

resistance starvation tolerance, VBNC formation, stress survival and combinations thereof.

7. A method according to any one of the preceding claims wherein the microorganism is a pathogenic or opportunistic strain of a bacterium.

8. A method according to any one of the preceding claims wherein the microorganism is selected from the group consisting of *Escherischia coli, Salmonella typhimurium, S. typhi, S. paratyphi, Shigella flexneri, Haemophilus influenza, Helicobacter pylori, Bacillus subtilis, Borrelia burgdorferi, Nessieria menignitidis, Yersinia pestis, Campylobacter jejuni, Vibrio cholerae, V. harveyi, V. parahaemolyiticus, V. vulnificus, Mycobacterium tuberculosis, Enterococcus faecalis, Streptococcus pneumoniae, S. pyogenes, Staphylococcus aureus, Clostridium perfringens, C. difficile, Porphorymonas gingivalis, Fusobacterium nucleatum, Yersinia enterocolitica, Y. pestis, Camplyobacteri jejuni, Heamophilus influenza, Shewenella putrafaceins, Bacillus anthracis. Bacillus anthracis, Neiseria gonorrheae, Borrelia burgdorferi* and *Klebsiella pneumonia*.

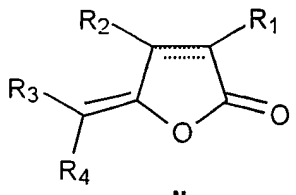
9. A method according to any one of the preceding claims wherein the virulence factor is a metalloprotease.

10. A method according to claim 9, wherein the virulence factor is a metalloprotease selected from the group consisting of VvpR of *V. vulnificus*, HA/P of *V. cholerae*, and EmpA from *V. parahaemolyticus*.

11. A method according to claim 9, wherein the virulence factor is metalloprotease production and the microorganism is *V. vulnificus*.

12. A method according to any one of the preceding claims wherein the compound is selected from the group consisting of 2, 3, 24, 26, 30, 34, 56, 57, 60, 63, 64, 70, 72, 73, 74, 75, 78, 80 85, 88, 91, 99, 102, 103, 105, 112, 113, 118, 124, 129, 139 as shown in Table 4.

13. A method of treating an animal to decrease the severity of symptoms resulting from bacterial infection, the method comprising administering to the animal an effective dose of a composition comprising a compound of general formula II:




II

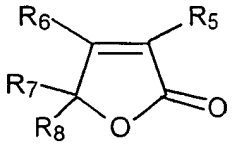
wherein $R_1$ and $R_2$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_3$ and $R_4$ are independently H, halogen, alkyl, aryl or arylalkyl, alkoxy, alkylsilyl;

$R_3$ or $R_4$ + $R_2$ s a saturated or an unsaturated cycloalkane; and

"-------" represents a single bond or a double bond,

or a compound of general formula III:



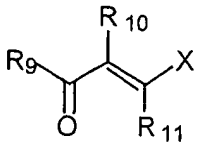

III

wherein $R_5$ and $R_6$ are independently H, halogen, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

$R_7$ and $R_8$ are independently H, halogen, hydroxy, alkyl, haloalkyl, dihaloalkyl, trihaloalkyl, aryl, arylalkyl, a hydroxy compound or derivative of a hydroxy compound;

$R_7$ or $R_8$ + $R_6$ is saturated or unsaturated cycloalkane.

or a compound of general formula IV




IV

wherein $R_{10}$ and $R_{11}$ are independently H, halogen, carboxyl, ester, formyl, cyano, alkyl, alkoxy, oxoalkyl, alkenyl, aryl or arylalkyl whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic;

X is a halogen;

$R_9$ is H, alkyl, alkenyl, alkynyl, alkene, alkyne, aryl, arylalkyl, whether unsubstituted or substituted, optionally interrupted by one or more heteroatoms, straight chain or branched chain, hydrophilic or fluorophilic.

14. A method according to any one of claims 1 to 12 when used for regulating biofilm formation by a microorganism.

15. A method according to claim 14, wherein the biofilm formation is AI-2 mediated.

16. A method according to claim 15, wherein the microorganism is selected from the group set out in claim 8.

17. A method according to any one of claims 14 to 16, wherein the compound is in a composition comprising a surfactant.

18. A method according to any one of claims 1 to 13 when used for cleaning a surface.

19. A method according to claim 18 wherein the surface is a hard surface, a woven surface, or a non-woven surface.

20. A method according to claim 18 or claim 19, wherein the microorganism is selected from the group set out in claim 8.

21. A method according to any one of claims 1 to 13, when used to treat or ameliorate dental disease or gingivitis.

22. A method according to claim 21, wherein the microorganism is selected from the group set out in claim 8.

23. A method according to any one of claims 1 to 13 when used in the treatment or prevention of dental caries.

24. A method according to claim 23, wherein the microorganism is selected from the group set out in claim 8.

25. A method according to any one of claims 1 to 13 when used for cleaning and disinfecting contact lenses.

26. A method according to any one of claims 1 to 13 when used for treating an medical indwelling device.

27. A method according to claim 26, wherein the device is selected from the group consisting of catheters, orthopedic devices and implants.

28. A method according to claim 13, wherein the animal is a shellfish.

29. A method according to claim 28, wherein the shellfish is an oyster and the microorganism is *Vibrio*.

30. A method according to claim 13, wherein the animal is a prawn.

31. A composition suitable for regulating biofilm formation comprising a compound of Formula II, III or IV as set out in claim 3 and a diluent or carrier.

32. A composition according to claim 31 further including a surfactant.

33. A dentifrice comprising a compound of Formula II, III or IV as defined in claim 3.

34. A mouthwash comprising a compound of Formula II, III or IV as defined in claim 3

35. A microbial disinfectant composition comprising an effective amount of a compound defined in claim 3.

36. A disinfectant according to claim 35 comprising a biocide or antibiotic.

37. A disinfectant coating comprising a compound of Formula II, III or IV as defined in claim 3.

38. A pharmaceutical composition comprising a compound of Formula II, III or IV as defined in claim 3 and a pharmaceutically acceptable diluent or carrier.

39. A person care product including a compound of formula II, III or IV as defined in claim 3.

40. A personal care product according to claim 39, selected from the group consisting of deodorants, soaps, shampoos, tampons and diapers.

41. A topical dressing or bandage comprising a compound of Formula II, III or IV as defined in claim 3.

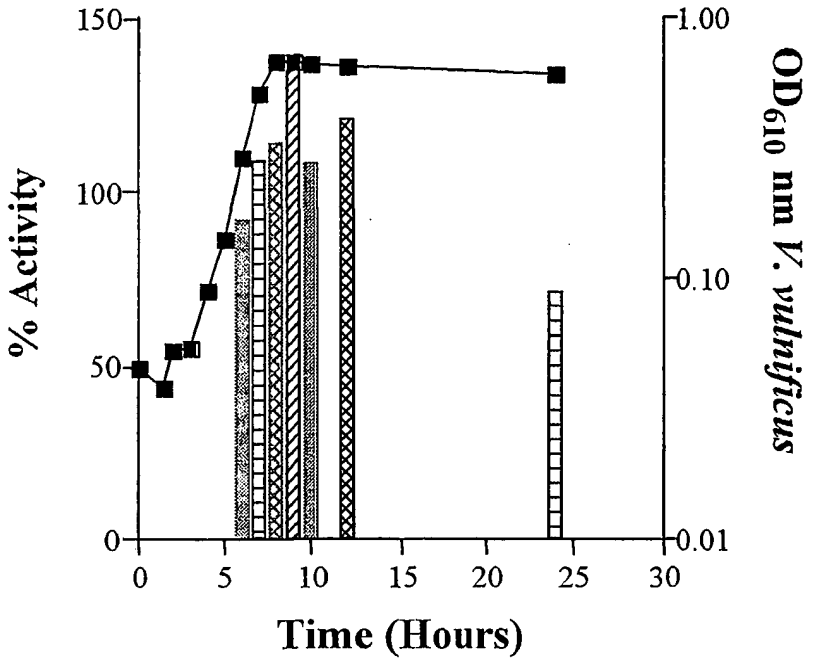
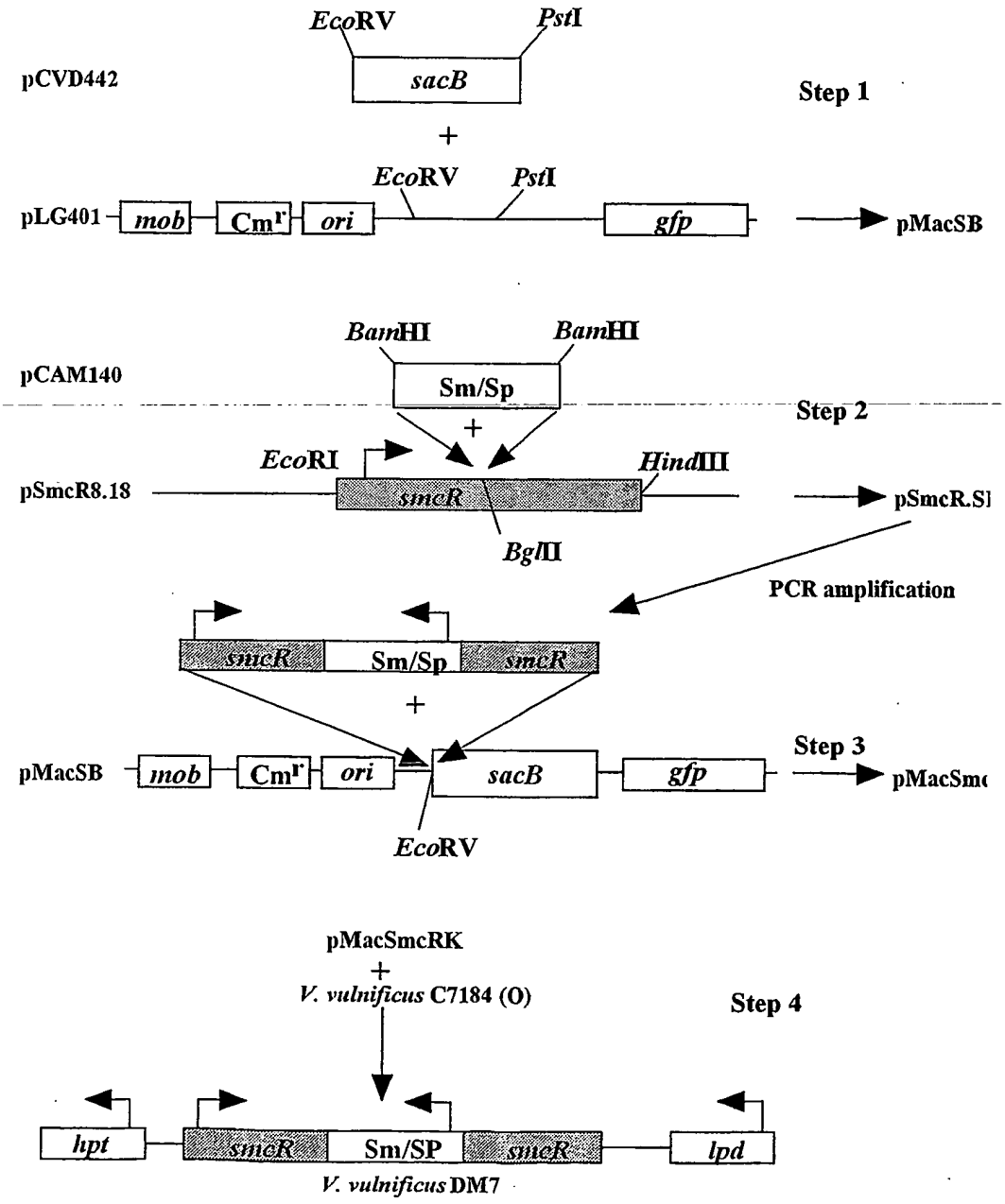
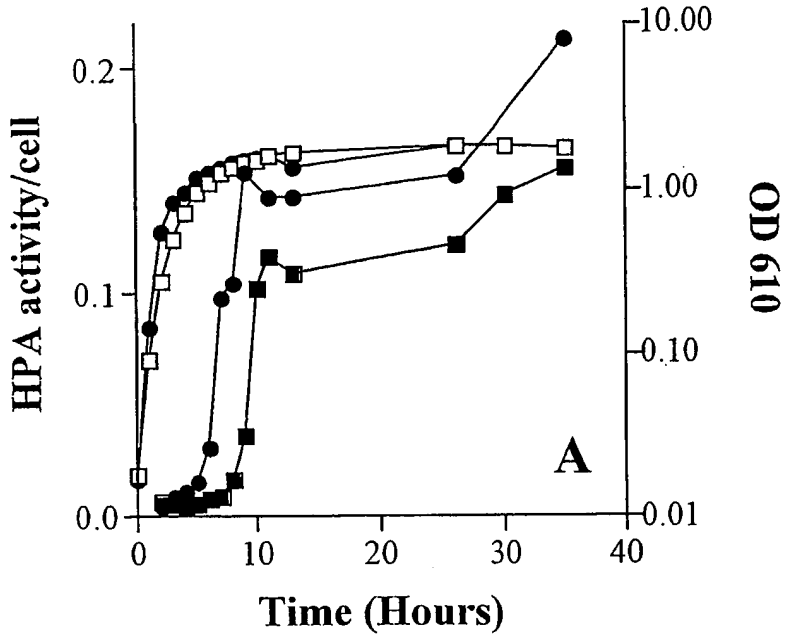

150
100
50
0
% Activity
1.00
0.10
0.01
OD610 nm V. vulnificus
0
5
10
15
20
25
30
Time (Hours)

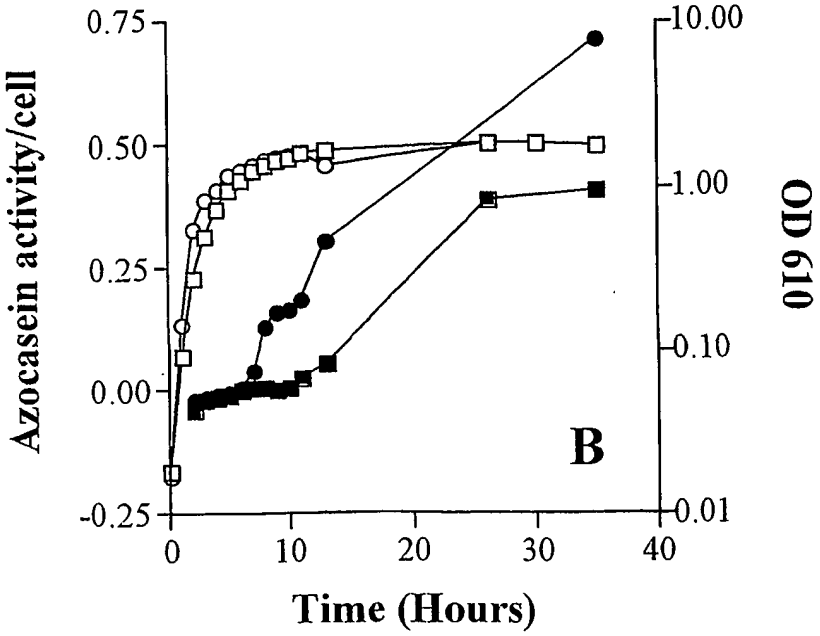
Fig 1

Fig 2




HPA activity/cell
0.2
0.1
0.0
0
10
20
30
40
Time (Hours)
10.00
1.00
0.10
0.01
OD 610
A

5


Azocasein activity/cell
0.75
0.50
0.25
0.00
-0.25
0
10
20
30
40
Time (Hours)
10.00
1.00
0.10
0.01
OD 610
B

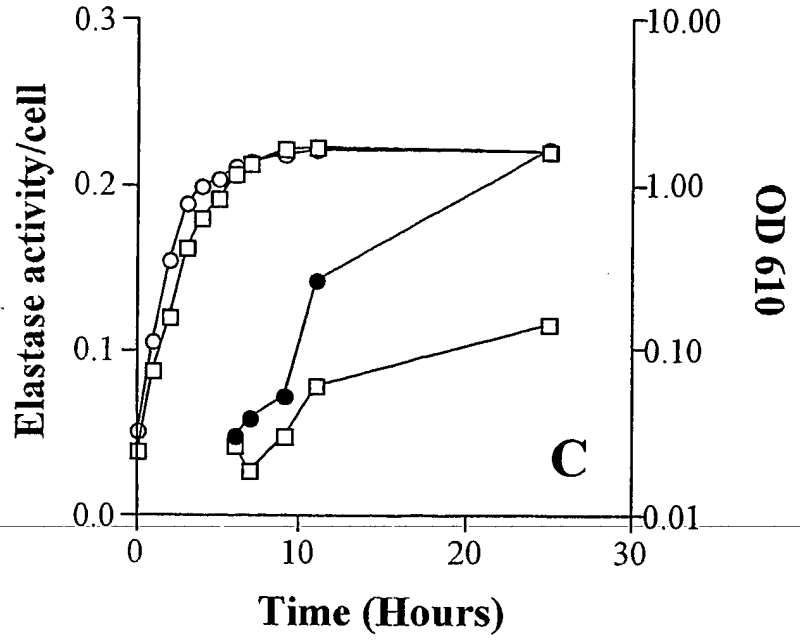



Fig 3

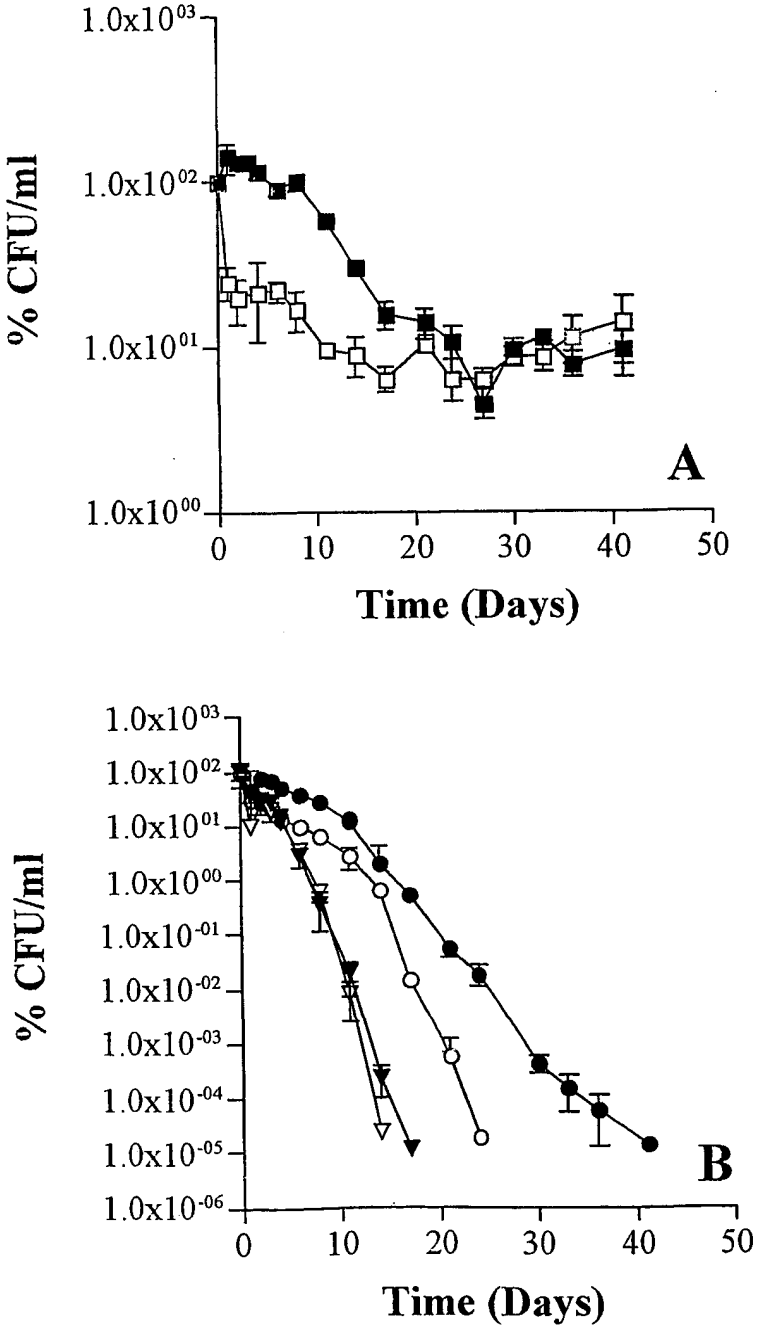



Fig 4

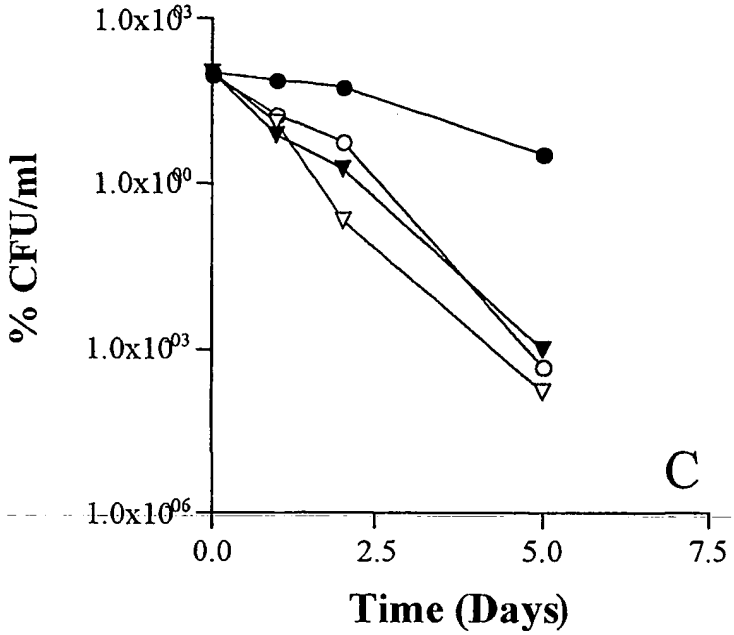

1.0x10^03
1.0x10^00
1.0x10^03
1.0x10^06
% CFU/ml
C
0.0
2.5
5.0
7.5
Time (Days)

Fig 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/01621

**A. CLASSIFICATION OF SUBJECT MATTER**

Int. Cl. [7]: A61K 31/365, 31/366, 31/121, 31/19, A61P 31/04

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

REFER ELECTRONIC DATABASE CONSULTED BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN File REGISTRY/CAPLUS: keywords: furanone, antimicrob, antibact, AI-2, micorganism, antifoul, disinfect, shellfish,dentrif, biofilm
MEDLINE keywords: AI-2, furanone

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | WO 0143739 A1 (UNISEARCH LIMITED) 21 June 2001<br>Entire document | 1-41 |
| P,X | WO 0176594 A1(UNISEARCH LIMITED)18 October 2001<br>Entire document | 1-41 |
| P,X | WO 0168090 A1 (UNISEARCH LIMITED) 20 September 2001<br>Entire document | 1-41 |

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 January 2002 | - 5 FEB 2002 |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustralia.gov.au<br>Facsimile No. (02) 6285 3929 | Authorized officer<br>**TERRY SUMMERS**<br>Telephone No : (02) 6283 3126 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU01/01621

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| P,X | WO 0168091 A1 (UNISEARCH LIMITED) 20 September 2001<br>Entire document | 1-41 |
| X | WO 9953915 A (UNISEARCH LIMITED) 28 October 1999<br>Entire document | 1-41 |
| X | WO 9954323 A (UNISEARCH LIMITED) 28 October 1999<br>Claims 21-24, pages 22-23, Figs 1,5 | 1-41 |
| X | WO 9905227 A (AQUACULTURE CRC LIMITED) 4 February 1999<br>Claims 1 and 9, Fig 1C | 1-41 |
| X | WO 9901514 A (AQUACULTURE CRC LIMITED) 14 January 1999<br>Claim 1 and 6, Fig 1C | 1-41 |
| X | WO 9629392 A (UNISEARCH LIMITED) 26 September 1996<br>Entire document, Figs 1-3 | 1-41 |
| X | WO 9601294 A (UNISEARCH LIMITED) 18 January 1996<br>Abstract, Figs 1-3 | 1-41 |
| X | Derwent Abstract Accession No. 20175Y, Class D22, DT2538771A1 (HENKEL & CIE GMBH) 17 March 1977<br>Abstract | 1-41 |
| X | McConnell O.J. *et al.*, Polyhalogenated 1-octene-3-ones, antibacterial metabolites from the red seaweed'*Bonnemaisonia asparagoides, Tetrahedron Letters*, 1977, No. 22, 1851-1854<br>Table 1 | 1-41 |
| X | Fenical W *et al.*, Antibiotics and antiseptic compounds from the family *Bonnemaisoniaceae (Florideophyceae)*, Proceedings of the International Seaweed Symposium, Volume Date 1977, 9th 1979, Issue Date 1979, 387-400<br>Abstract, pp 389-391 | !-41 |
| X | Manefield M. *et al.*, Evidence that halogenated furanones from *Delisea pulcha* inhibit acylated homoserine lactone (AHL)- mediated gene expression by displacing the AHL signal from its receptor protein, *Microbiology*, 1999, 145, 283-291<br>Entire article, especially, abstract and Fig 1 | 1-41 |
| X | Bassler B. L., How bacteria talk to each other: regulation of gene expression by quorum sensing, *Current Opinion in Microbiology*, 1999, 2, 582-587<br>Entire article | 1-2 |
| X | Dal Pozza A. et al. α,β- Unsaturated γ-lactones correlations between lipophilicity and biological activity, Arzneim.-Forsch/ Drug Res, 1979, 29(I), No 6, 877-882<br>Abstract, page 877 column 2 | 1-46 |
| P,X | McDougald D. *et al.* SmcR-dependent regulation of adaptive phenotypes in *Vibrio vulnificus, J Bacteriology*, January 2001, 183(2), 758-762<br>Entire article, page 760 column 1 lines 39-46 | 1-41 |

**INTERNATIONAL SEARCH REPORT**
Information on patent family members

International application No.
**PCT/AU01/01621**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member | | | | |
|---|---|---|---|---|---|---|
| WO | 200143739 | AU | 200123284 | | | |
| WO | 200176594 | AU | 20006812 | | | |
| WO | 200168090 | AU | 20006292 | | | |
| WO | 200168091 | AU | 20006290 | | | |
| WO | 9953915 | AU | 33224/99 | EP | 1071416 | |
| WO | 9954323 | AU | 33225/99 | EP | 1071677 | |
| WO | 9905227 | AU | 80942/98 | EP | 998533 | NO 20000315 |
| WO | 9901514 | AU | 80943/98 | EP | 996681 | NO 20000014 |
| WO | 9629392 | AU | 49996/96 | BR | 9607661 | CA 2215797 |
| | | CN | 1185173 | EP | 815201 | NZ 303630 |
| WO | 9601294 | CN | 1156471 | AU | 28750/95 | CA 2192955 |
| | | EP | 769039 | HK | 1001287 | NZ 289025 |
| | | US | 6060046 | | | |
| DE | 2538771 | NONE | | | | |

END OF ANNEX